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Sara Trifari

**The role of Wiskott-Aldrich Syndrome protein in
activation and function of human T cells**

Degree of Doctor of Philosophy in Molecular and Cellular Biology

May 2006

**Sponsoring establishment: San Raffaele Telethon Institute for Gene Therapy
(HSR-TIGET)**

Milan, Italy

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DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were generated by myself, with the exception of the RNase protection assays, which were performed in collaboration with Giovanni Sitia and Luca Guidotti at DIBIT, San Raffaele Scientific Institute. The lentiviral vectors, used in this study, were generated by Antonia Follenzi and Lucia Sergi-Sergi in the laboratory of Luigi Naldini at HSR-TIGET.

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“Wiskott-Aldrich Syndrome Protein regulates lipid rafts dynamics during immunological synapse formation” *Immunity* 17, 157 (2002).

Dupré L., Trifari S., Follenzi A., Marangoni F., Lain de Lera T., Bernard A., Martino S., Tsuchiya S., Bordignon C., Naldini L., Aiuti A., and Roncarolo M.G.

“Lentiviral vector-mediated gene transfer in T cells from Wiskott-Aldrich syndrome patients leads to functional correction” *Mol Ther* 10, 903 (2004).

Trifari S., Sitia G., Aiuti A., Scaramuzza S., Marangoni F., Guidotti L.G., Martino S., Saracco P., Notarangelo L.D., Roncarolo M.G., and Dupré L.

“Defective Th1 cytokine gene transcription in CD4⁺ and CD8⁺ T cells from Wiskott-Aldrich Syndrome patients” *Submitted to Journal of Immunology* (under 3rd revision) (2006).

Marangoni F., Trifari S., Scaramuzza S., Battaglia M., Roncarolo M.G., and Dupré L.

“WASP regulates activation and suppressor activity of human and murine CD4⁺CD25⁺FoxP3⁺ natural regulatory T cells” *In preparation* (2006).

ABSTRACT

Wiskott-Aldrich Syndrome (WAS) is a X-linked genetic disease caused by mutation in the gene encoding for the Wiskott-Aldrich Syndrome protein (WASP). WASP is specifically expressed in hematopoietic cells, where it regulates the reorganization of actin cytoskeleton in response to extracellular stimuli. WAS is characterized by micro-thrombocytopenia, eczema, immunodeficiency and high susceptibility to autoimmune disorders and haematological malignancies. Although WASP deficiency affects the function of several haematopoietic cells, T cell dysfunction plays a predominant role in WAS pathogenesis. Our study focused on the functional and molecular analysis of different effector T cell subsets from WAS patients and on a possible defect in peripheral tolerance.

We found that WASP lowers the threshold for T cell proliferation, induced by TCR/CD28 triggering. Restoration of WASP expression mediated by lentiviral vectors gene transfer fully reconstitutes TCR-driven proliferation. Furthermore, we showed that WASP regulates the levels of the ganglioside GM1 upon TCR/CD28 triggering. In addition, WASP deficiency selectively impairs the production of Th1 cytokines, after TCR/CD28 triggering in CD4⁺ and CD8⁺ T cells from WAS patients, as a consequence of reduced Th1 cytokine gene transcription. Reduced expression of IL-2 and IFN- γ mRNA in WAS T cells is associated with reduced nuclear levels of NFAT, and in WAS CD4⁺ T cells also with defective induction of T-bet transcription factor.

The function of natural regulatory T cells (nTreg) from WAS patients was also investigated, showing that WASP deficiency impairs the suppressive activity of nTreg cells.

This study clarifies the role of WASP in T cell activation and effector functions, and suggests that dysfunctions in regulatory T cells can be involved in the pathogenesis of WAS.

1 INTRODUCTION

1.1 Wiskott-Aldrich Syndrome: an overview

Wiskott-Aldrich Syndrome (WAS) is an X-linked genetic disease, characterized by micro-thrombocytopenia, eczema, immunodeficiency and increase susceptibility to haematological malignancies and autoimmune disorders. The syndrome was described in 1937 by Wiskott (Wiskott, 1937) and its features of recessive, sex-linked, disorder were defined by Aldrich about 20 years later (Aldrich et al., 1954). Initially, the disease was linked with altered expression of sialophorin (also called CD43) on the surface of T lymphocytes and platelets (Parkman et al., 1981), but this alteration was then showed not to be the primary cause of the disease. Indeed, the gene responsible for WAS was cloned in 1994 (Derry et al., 1994) and the product of this gene, a 502 amino acid protein, was named WASP (Wiskott-Aldrich Syndrome Protein). Later on, it was discovered that mutations in the WAS gene could lead to a milder form of the disease, called XLT (X-linked thrombocytopenia), which is characterized by thrombocytopenia and eczema but with an overall preservation of the immune functions. (Notarangelo et al., 2002; Villa et al., 1995). Both WAS and XLT are due to mutations which abolish or reduce WASP expression and function. Recently, the picture has been made even more complex by the discovery of patients, in which mutations leading to a constitutive activation of WASP are the cause of a distinct disease, named XLN (X-linked Neutropenia) (Devriendt et al., 2001). The most relevant clinical manifestation of XLN is a severe neutropenia and monocytopenia, which is the cause of major bacterial infections, associated to a block in the maturation of myeloid lineage at the premyelocyte/myelocyte stage.

WAS has been, and continues to be, the object of numerous studies aimed to unravel the disease pathogenesis and the role of WASP in the immune system. However, much

remain to be done to fully clarify the cellular and molecular mechanisms underlying WAS.

1.2 Wiskott-Aldrich Syndrome Protein (WASP): structure and function

The gene responsible for WAS was initially found to be located in the Xp11.22-11.23 region (Peacocke and Siminovitch, 1987) and it was subsequently isolated by positional cloning (Derry et al., 1994). The WAS gene is constituted by 12 exons and encodes for a protein of 502 amino acids. WASP was initially hypothesized to be a transcription factor, based on the presence of poly-proline stretches, an highly acidic domain at the C-terminus and a putative nuclear localization signal (Dingwall and Laskey, 1991)(PADKKRSGKKKISK, residues 222-235). Next studies revealed instead that WASP is the prototype of a family of proteins, which controls actin dynamics. These proteins are largely conserved throughout evolution and can be divided into two groups, according to the structure and mode of activation. The first group comprises WASP (Derry et al., 1994), which is expressed in the haematopoietic system, and the more widely expressed N-WASP (Miki et al., 1996). The second group comprises three Scar/WAVE proteins: WAVE1, WAVE2 and WAVE3 (Bear et al., 1998; Miki et al., 1998b; Suetsugu et al., 1999). WASP and WAVE proteins share a similar organization in the C-terminal portion of the protein, while the structure of the N-terminus is much more divergent. A schematic representation of WASP and related proteins is shown in Figure I.

Figure I

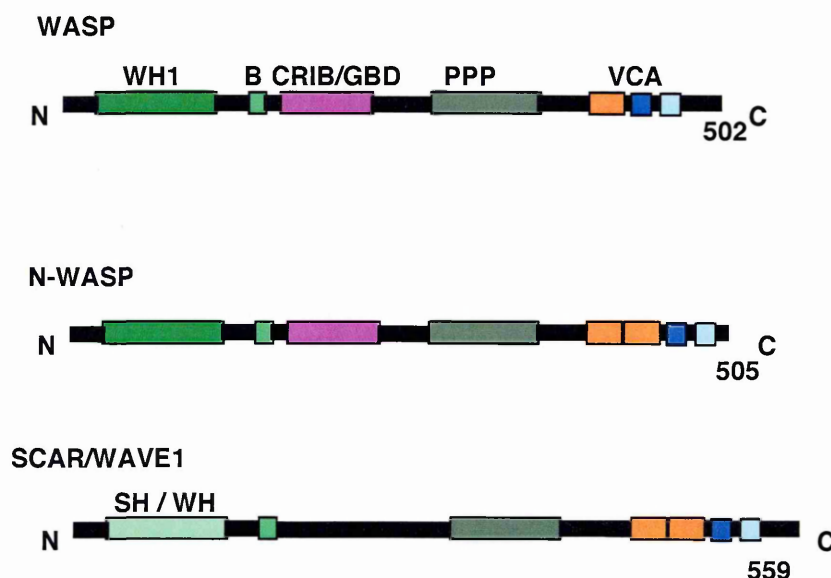


Figure I.

Structure of Wiskott-Aldrich Syndrome protein (WASP), neural (N)-WASP and Suppressor of cAMP receptor/WASP family verprolin homologous (SCAR/WAVE) proteins. WH1: WASP homology domain 1; B: basic region; CRIB/GBD: Cdc42 and Rac interactive binding domain/GTPase binding domain; PPP: proline-rich region; V: verprolin; C: cofilin; A: acidic. SH/WH: SCAR homology/WAVE homology domain.

In the C terminal part of these proteins is located the VCA (verprolin-homology, central [or cofilin] and acidic) domain. The V domain binds actin monomers (Miki et al., 1998a; Rohatgi et al., 1999), while the CA domain binds a large multi-molecular complex, named Arp2/3, which is one of the most important known activator of actin polymerization (Welch et al., 1997). Although the VCA domains of WASP and Scar/WAVE proteins have similar affinity for Arp2/3 binding, each domain has a distinct efficacy in driving actin polymerization. The N-terminal portion (which actually comprises >85% of the entire amino acid sequence) of WASP and N-WASP contains a WH1 (WASP-homology 1) domain, a basic region (B), a GTP-ase binding domain

(GBD/CRIB) and a proline-rich region (P). The WH1 domain, which comprises the first 150 amino acids of WASP was initially thought to mediate the binding to phosphatidylinositol(4,5)-biphosphate [PI(4,5)P₂] (Imai et al., 1999; Miki et al., 1996), also in view of its fold analogy with the pleckstin homology (PH) domain, which binds to phosphoinositides (Rebecchi and Scarlata, 1998). Subsequently, the ability to bind PIP₂ was rather attributed to the B domain (Rohatgi et al., 2000). The WH1 domain mediates the binding to WIP (WASP interacting protein) (Ramesh et al., 1997) while the GBD/CRIB domain binds to GTP-bound Cdc42 (Kolluri et al., 1996; Miki et al., 1998a; Symons et al., 1996). Cdc42 is a member of the small Rho GTPase family, which also comprises Rho and Rac (Bishop and Hall, 2000). These proteins control a variety of cellular processes, including cell migration, cell morphology, endocytosis and adhesion, in part through the activity of WASP/WAVE proteins. The respective regulation of WASP/N-WASP by Cdc42 and of WAVE proteins by Rac is one of the main differences in the specificity of these two groups of related proteins (Bompard and Caron, 2004). While WAVE proteins are constitutively active (Miki et al., 1998b), regulation of WASP/N-WASP activity is exerted at the level of conformational changes. Indeed, these proteins were shown to exist, in resting conditions, as auto-inhibited monomers, in which inhibition is achieved through intra-molecular binding of the B/GBD to the VCA domain (Kim et al., 2000). The classic model of WASP/N-WASP function implies that extracellular stimuli activate WASP/N-WASP by relieving the conformational inhibition through the binding of phosphatidylinositol 4,5-biphosphate (PI[4,5]P₂) and of activated Cdc42 (Higgs and Pollard, 2000; Kim et al., 2000; Prehoda et al., 2000; Rohatgi et al., 2000). However, recent findings led to further clarify the precise molecular mechanisms of WASP/N-WASP activation and underscored the importance of WIP in this process. N-WASP activation by Cdc42 was shown to require the binding of Toca-1, a member of the PCH protein family, which is highly conserved in eukaryotes (Ho et al., 2004). Given

the high homology between WASP and N-WASP, it is likely that a similar mechanism accounts also for WASP activation. In resting lymphocytes more than 95% of WASP is bound to WIP (Sasahara et al., 2002). This interaction is important for regulation of both localization and activation of WASP. Indeed, many missense mutations causing WASP were predicted to disrupt WASP-WIP interaction (Stewart et al., 1999; Volkman et al., 2002; Zhu et al., 1997). It was therefore proposed that binding of Cdc42 and Toca-1 to the (N-)WASP-WIP complex would lead to dissociation from WIP and (N-)WASP activation, a process favoured by the binding of PIP2.

Moreover, a variety of SH3-containing proteins, including Src, Fyn, PI3K p85, PLC γ 1, Nck, Itk, profilin, VASP and WISH, were found to bind the poly-proline rich region of WASP/N-WASP in either *in vivo* or in cell-free systems (reviewed in (Imai et al., 2003; Takenawa and Miki, 2001)). This region regulates both the subcellular localization and the activation of WASP/N-WASP in response to extracellular stimuli. A final level of regulation of WASP/N-WASP is exerted by phosphorylation. Indeed, the VCA domain of these proteins is serine-phosphorylated by casein kinase 2 and this phosphorylation was shown to increase the actin-nucleating activity (Cory et al., 2003). Moreover, both WASP and N-WASP can be tyrosine phosphorylated on a residue, which is located into the GBD/CRIB domain. This post-translational modification increases the ability of WASP to promote actin polymerization (Cory et al., 2002). An integrated model of regulation of WASP activation and localization is shown in Figure II. The overall role of WASP/WAVE proteins is to create a branched network of actin filaments. Different models of initiation of actin polymerization have been proposed to explain the mechanisms of cytoskeleton remodelling. Arp2/3 complex seems to have a non-redundant role in this process, by inducing polymerization of new actin filaments on the side of pre-existing filaments, thus creating a branching network of actin. The activity of Arp2/3 complex was shown to contribute to a variety of cellular functions, including

changing of cell shape, motility, endocytosis and phagocytosis (Welch and Mullins, 2002).

Thus, WASP (in the immune system) as well as N-WASP (in other tissues) are positioned, in a well-orchestrated manner, at the cross-road of multiple signalling pathways. As a result, WASP family proteins integrate extra-cellular stimuli and translate them into remodelling of the actin cytoskeleton, and, possibly, into regulation of other downstream targets.

Figure II

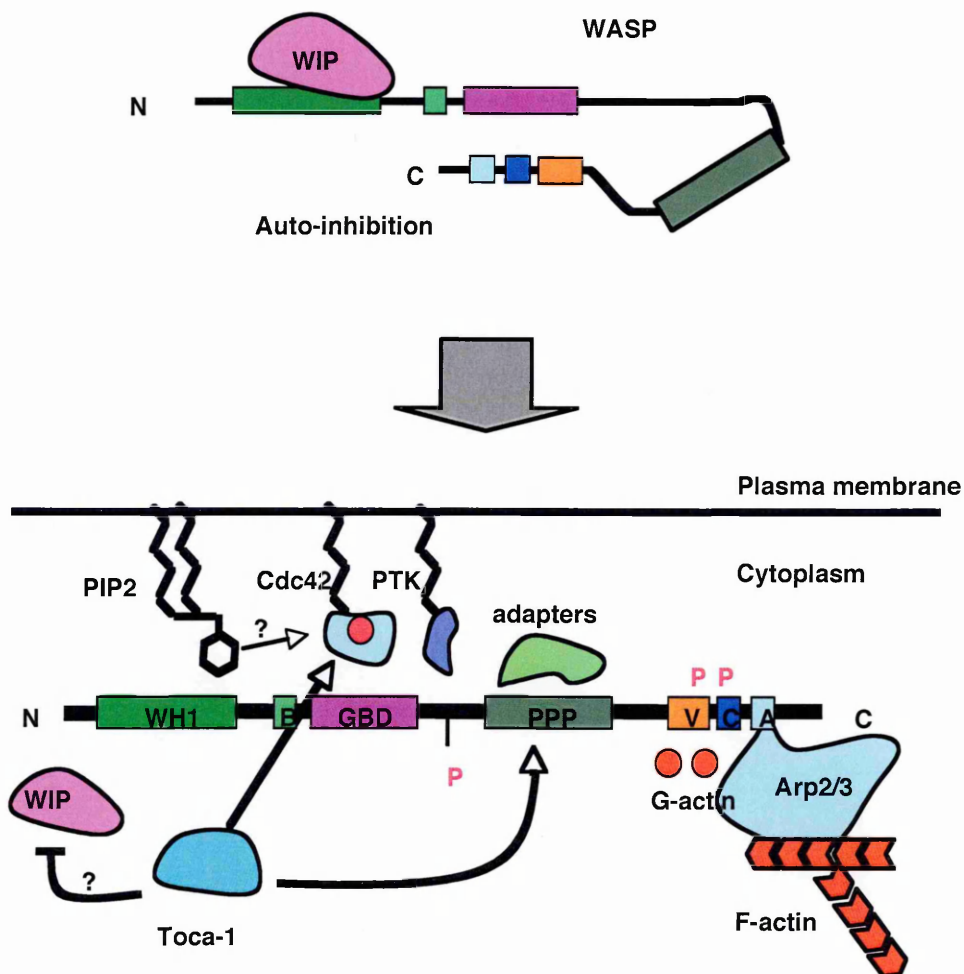


Figure II. In resting cells (upper panel) WASP exists in a monomeric auto-inhibited conformation, maintained by an intra-molecular binding between the CRIB and the VCA domain. The majority of WASP is bound to WIP, when inactive. Upon triggering of cell surface receptors (low panel), a cascade of signalling events leads to the binding of GTP-bound Cdc42 to the CRIB domain. This binding, together with the binding of Toca-1 and PIP2, relieves the inhibition and allows the VCA domain to interact with the Arp2/3 complex. Tyrosine and serine phosphorylation by Src kinase and CKII, respectively, enhances WASP activity.

1.3 WASP expression during haematopoietic differentiation

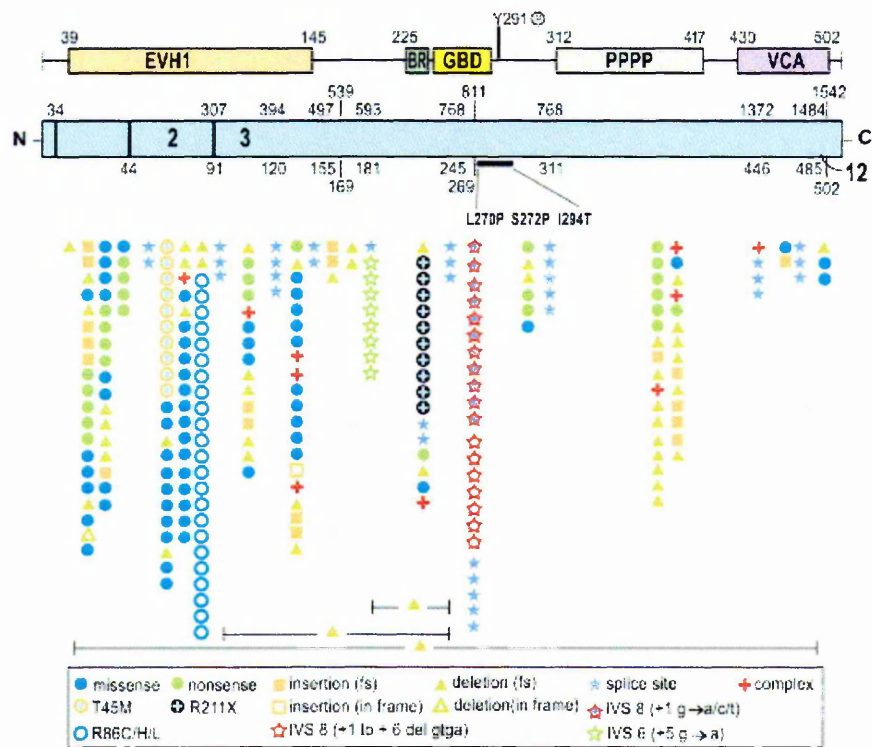
The first evidence of the hematopoietic-restricted pattern of WASP expression came from the observation that WASP mRNA was expressed in human T and B cell lines, as well as in the spleen and the thymus, but not in other non-haematopoietic tissues (Derry et al., 1994). This pattern was consistent with the clinical manifestations of WAS, which affects the haematopoietic system. In addition, WASP mRNA was found to be expressed already in human CD34⁺ progenitor cells and its expression was maintained throughout the differentiation into all the different lineages (Parolini et al., 1997). Accordingly, WASP was shown to be expressed in CD34⁺ cells and in various haematopoietic cells lineages (Zhu et al., 1997), suggesting that WASP could be involved in early differentiation and survival of haematopoietic precursors. This possibility was confirmed by the observation that in healthy female carriers of WAS a non-random inactivation of the X chromosome was found in all haematopoietic lineages, including progenitor CD34⁺ cells, while in the same subjects a random inactivation of the X chromosome was found in non-haematopoietic tissues (Wengler et al., 1995). This skewed X inactivation could result from a selection of the cells which have inactivated the mutated X chromosome, due to an *in vivo* selective advantage (Belmont, 1996). However, some cases of females subjects with clinical symptoms of WAS were reported. In some cases, a random X chromosome inactivation was observed in the haematopoietic compartment (Lutskiy et al., 2002). The involvement of WASP in haematopoietic progenitor differentiation was further confirmed by the observation that CD34⁺ cells isolated from WAS patients has a reduced ability to give rise to both megakaryocytes, granulocyte-macrophages and erythroid colonies *in vitro*, as well as to a reduced ability to form pro-platelets (Kajiwara et al., 1999). Interestingly, normal colony numbers but defective pro-platelets formation was observed starting from CD34⁺ cells isolated from XLT patients, (Kajiwara et al., 1999). These data support the possibility that WASP

could confer a selective advantage early during haematopoietic differentiation. Studies from one WASP knock-out mouse model showed that transplanted WASP⁺ bone marrow (BM)-derived cells had a higher ability to home to BM compared to WASP⁻ cells (Lacout et al., 2003). In the same study, skewed X-chromosome inactivation was observed in BM cells of heterozygous female mice and was related to an advantage for WASP⁺ cells to migrate from fetal liver to BM. Thus, WASP seems to be required both for migration and for proliferation/survival of haematopoietic progenitors.

1.4 WAS and XLT: pathology

1.4.1 Genotype-phenotype correlation

More than 300 unique WAS gene mutations were identified and collected in a WAS database (<http://homepage.mac.com/kohsukeimai/wasp/WASPbase.html>). These mutations are scattered throughout the entire length of the WAS gene, although some hotspots have been identified and found to be associated to either WAS or XLT (Jin et al., 2004) (Figure III).



Ochs HD. and Thrasher AJ. *J. Allergy Clin. Immunol.* (2006)

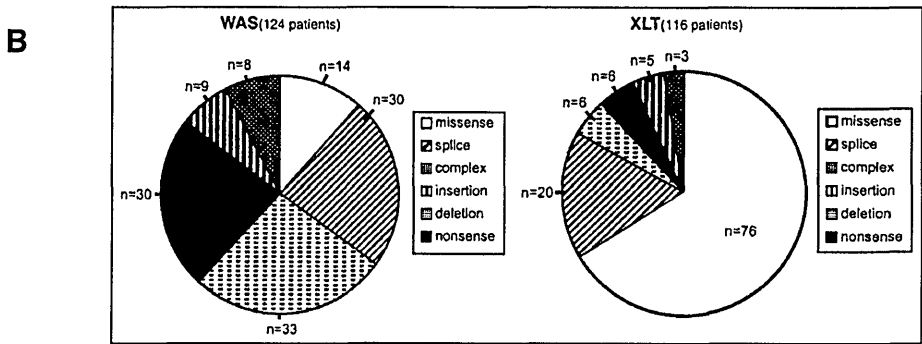
Recent studies, performed on large cohorts of patients, showed a strong genotype-phenotype correlation. Indeed, mutations, which abolish WASP expression (usually deletion/insertion, nonsense mutations and splice site mutations) are associated with a severe clinical phenotype (full blown WAS), a disease score of 3-5 and a life expectancy below 20 years of age. On the contrary, missense mutations, which result in residual expression of a full-length point-mutated WASP, are associated with a mild

clinical phenotype (XLT/WAS), corresponding to a disease score of 0.5-2 and a longer life expectancy (Imai et al., 2004; Jin et al., 2004) (Figure IV). All patients harbouring mutations in the *WAS* gene are thrombocytopenic and their platelets are smaller than the normal, although in some patients with mutations resulting in a single amino acid substitution, the thrombocytopenia is intermittent (Notarangelo et al., 2002). Both WAS and XLT patients suffer from haemorrhages, which can be lethal (generally in case of intra-cranial haemorrhages). Eczema or atopic dermatitis are another frequent complications of WAS and XLT and can be, in some cases, resistant to conventional therapies (Imai et al., 2004).

Figure IV WAS gene mutations and clinical score

A	XLT		WAS		
	1	2	3	4	5
Thrombocytopenia	+/-	+	+	+	+
Eczema	-	+/-	+	++	++/-
Immunodeficiency	-	+/-	+	++	++/-
Autoimmunity or malignancy	-	-	-	-	+

Zhu, Blood 1997,90:2680
 Notarangelo, Blood 2002,99:2268
 Jin, Blood 2004,104:4010



Ochs HD and Notarangelo LD.
 2005, Curr Op Hematol, 12:284

Figure IV. Clinical manifestations and disease score in individuals with mutations in the *WAS* gene (A). Mutations identified in 124 patients with classic WAS and in 116 patients with XLT (B). WAS phenotype is usually associated to complex mutations, which abolish WASP expression, while XLT is mainly associated to missense mutations.

1.4.2 Infections

The vast majority of WASP-negative patients have a history of recurrent infections, which can be life-threatening (Imai et al., 2004; Sullivan et al., 1994). Infections in WAS patients are due to bacteria, virus and fungi. Bacterial infections are generally due to encapsulated bacteria and can affect either the airways (causing otitis media, pneumonia) the gut (causing enterocolitis), the skin, the urinary tract, the central nervous system, or they also can be disseminated. Viral infections are generally due to herpes simplex virus (HSV), cytomegalovirus (CMV), Epstein Barr virus (EBV) and *Molluscum Contagiosum*. Among fungal infections, *Candida*, *Aspergillus* and *Pneumocystis carinii* have been reported in WAS patients.

1.4.3 Serum immunoglobulins

Serum immunoglobulin levels are altered in WAS, with IgM levels being generally lower than the normal range (although in some patients IgM levels were found to be higher than the normal range and this was associated with autoimmune manifestations and poor prognosis (Dupuis-Girod et al., 2003)). IgG levels are normal, while IgA and in particular IgE levels are often elevated. Although eczema has been associated to high IgE levels in the serum, in other immunodeficiencies, this seems not to be the case in WAS patients (Imai et al., 2004). An inability to produce Abs against polysaccharide Ags was classically found in WAS patients (Ayoub et al., 1968) and it was proposed to be one of the basis for the susceptibility to infections caused by encapsulated bacteria. The reason for this deficiency is still unclear. Although WAS patients were shown to produce low levels of Abs (including IgM and IgG) directed against polysaccharide Ags of various sources (*Streptococcus pyogenes*, *Haemophilus Influentiae* capsule, phosphocoline and blood group B), this was not due to an overall reduction in the levels of circulating IgG2 (Nahm et al., 1986), which is the subclass generally elicited by carbohydrate Ags (Siber et al., 1980).

1.4.4 Haematological malignancies

In addition to infections, a propensity to the development of lymphoreticular malignancies has been reported among full blown WAS patients (Imai et al., 2004; Shcherbina et al., 2003; Sullivan et al., 1994). These malignancies were found to occur in 13-22% of the patients and are mainly constituted by EBV-positive lymphoma and myelodysplasia. EBV-positive lymphoma has been frequently observed in patients with immunodeficiency, since in these patients the immune-surveillance against EBV, which includes production of neutralizing Abs, NK lytic activity, production of EBV-specific CTLs, is compromised. In particular, in WAS patients high levels of circulating Abs directed against viral capsid antigens (Ag) and low levels of anti-EBV determined nuclear antigens (EBNA) were reported (Okano et al., 1984). However, it is likely that immunodeficiency is not the only reason of high incidence of lymphoma, as a splice site mutation, associated with a mild clinical phenotype, was shown to be associated to a high risk of lymphoma (Shcherbina et al., 2003). One case of atypical lymphoproliferative disorder was also reported in a WAS patient (Ma et al., 2005), who showed reactive lymph node hyperplasia associated to altered nodal architecture, hepatosplenomegaly and dysgammaglobulinemia.

1.4.5 Autoimmunity

In addition to malignancies, another severe complication associated to WAS is autoimmunity. The reported frequency of WAS patients presenting autoimmunity were 24%, 40% and 72% in three independent studies (Dupuis-Girod et al., 2003; Imai et al., 2004; Sullivan et al., 1994). Autoimmune manifestations included haemolytic anemia, cutaneous vasculitis, inflammatory bowel disease, arthritis and IgA nephropathy. This last manifestation has been proposed to be due to alteration of the O-glycosylation pattern of IgA (Allen and Feehally, 2000), rather than to the production of specific autoreactive Abs (Imai et al., 2004). Interestingly, autoimmunity did not necessarily

correlate with disease severity, as it was present with a similar incidence in WASP⁺ and WASP⁻ patients (Imai et al., 2004). Mechanisms of WAS-associated autoimmunity are not clear, although inflammation due to the persistence of pathogen-derived Ag or alterations of either central or peripheral tolerance could be responsible for these complications.

Because of the high variability of disease severity, in patients with mutations in the *WAS* gene, these studies of genotype-phenotype correlation are of great importance, as they showed that WASP expression could be considered a useful prognostic indicator, at least for the occurrence of severe infections and haemorrhages. Therefore, analysis of WASP expression, when standardized, may help in the management of patients and in the evaluation of the best therapeutic option.

1.5 WAS and XLT: cellular defects

1.5.1 Platelets defects in WAS and XLT

WAS and XLT patients have thrombocytopenia and small platelets and, as a result, tendency to hemorrhagic diathesis. Bleeding is still one of the major causes of death among WAS and XLT patients (Imai et al., 2004). Platelets count in these patients vary from 6000 to 70000/ μ l (Imai et al., 2004). Clinical manifestations of bleeding were reported in 84% of WAS patients (based on a retrospective study) and range from not life-threatening, including petechiae, purpura, epistaxis, oral bleeding, to life-threatening, including gastrointestinal or intracranial haemorrhages (Sullivan et al., 1994). Although the incidence of life-threatening haemorrhages were found to be higher and to have a worse outcome in patients harbouring severe WAS gene mutations than in patients with mild WAS gene mutations, the presence or absence of WASP expression in lymphocytes did not to correlate with the extent of platelets abnormalities (Imai et al., 2004). The precise function of WASP in platelets has not been determined yet. Platelet activation was found to be defective in WAS/XLT patients. In particular, induction of CD62P (P-selectin) and GPIIb/IIIa (receptor for fibrinogen or CD41) in response to thrombin was reduced (Semple et al., 1997). Although WASP was shown to be phosphorylated in platelets upon collagen binding to the collagen receptor, Glycoprotein VI, possibly by the Tec kinase Btk (Oda et al., 1998), contrasting data were collected regarding the ability of WASP-deficient platelets to aggregate in response to collagen (Grottum et al., 1969; Kuramoto et al., 1970). The nature of the thrombocytopenia in WAS/XLT patients is still a matter of debate. Although WAS patients were reported to have normal or even high number of megakaryocytes in the BM (Grottum et al., 1969; Imai et al., 2004; Ochs et al., 1980), contrasting data have been obtained regarding the ability of BM-derived CD34⁺ cells from WAS/XLT patients to differentiate *in vitro* into megakaryocytes and to form pro-platelets (Haddad et al., 1999; Kajiwara et al., 1999). Overall, these data

suggest that ineffective thrombopoiesis is probably not the major cause of thrombocytopenia in WAS/XLT patients. Accordingly, normalization of both platelets count and size were generally observed in splenectomized patients (Corash et al., 1985), suggesting that platelets destruction, occurring in the spleen, has a major role in determining thrombocytopenia in WAS/XLT patients. Indeed, extensive co-localization of platelets and macrophages was observed in the spleen of WAS patients (Shcherbina et al., 1999b). Increased phagocytosis of WAS platelets by macrophages could result either from increased phosphatidylserine (PS) exposure on the outer leaflet of platelets plasma membrane or to binding of specific Ab to platelets. However, this second mechanism seems to be more relevant in the cases of relapse of thrombocytopenia, which may occur after splenectomy in WAS patients (Dupuis-Girod et al., 2003). Although WASP acts downstream of Cdc42 in mediating Arp2/3 complex activation and actin polymerization, normal Arp2/3 activation and localization, as well as normal platelets morphology and lamellipodia formation, were reported in platelets from WAS patients (Falet et al., 2002). Although some of these events (such as lamellipodia formation) are likely to be regulated by WAVE2 in platelets (Oda et al., 2005), these data suggest that WASP deficiency could affect the integrity of platelets more than their function. It is conceivable that alteration in the structure of platelets determines the physical trapping or destruction of normal-sized platelets during the transmigration in the splenic vasculature (Burns et al., 2004a). Since murine platelets are normally smaller than the human ones, this hypothesis could explain why in the mouse models of WAS thrombocytopenia is less severe than in WAS patients. A recent study has shown that in the BM of WASP knock-out mice the number of megakaryocytes is higher than in wild type mice. The morphology of WAS BM megakaryocytes is altered, as they do not have podosomes, which are adhesive structures, composed by a central core of actin and actin-associated proteins, surrounded by a ring of vinculin and linked to integrins (Tarone et al., 1985). In the same study,

ectopic formation of proplatelets in the WAS BM has been reported, probably due to the lack of a negative feedback transmitted from the BM environment to the megacaryocytes through the binding of fibrillar collagen I to $\alpha 2\beta 1$ integrin. These results suggest that in the absence of WASP premature platelets shedding could occur in the BM and therefore this phenomenon could contribute to WAS-associated thrombocytopenia (Sabri et al., 2006).

Interestingly, mutations which allow residual expression of WASP in lymphocytes, resulted in lack of WASP expression in platelets (Shcherbina et al., 1999a). This could explain why platelets abnormalities are present both in XLT and WAS patients and suggest that the stability of a point mutated WASP could be reduced with respect of the wild type proteins.

1.5.2 Defects in myeloid cells

The role of WASP in the organization of actin cytoskeleton during a variety of cellular functions suggests that a component of WAS-associated immunodeficiency could result from defective cell trafficking, pathogen uptake and clearance (Burns et al., 2004a). Chemotaxis (defined as directed migration along a gradient of a chemoattractant molecules) of monocytes isolated from the peripheral blood of WAS patients was reduced in response to a variety of stimuli, including MCP-1 (monocyte chemoattractant protein 1) and FMLP (formyl-methionil-leucyl-phenylalanine) (Altman et al., 1974; Badolato et al., 1998).

The deficiency of WASP in human macrophages led to the complete absence of podosomes (Linder et al., 1999). Podosomes are highly dynamic structures that, during chemotaxis, re-localize behind the leading edge of the cell. In WASP-deficient macrophages not only podosomes are missing but also filopodia and lamellipodia, whose formation depends on Cdc42 and Rac, respectively, are dispersed around the cell, instead of being polarized behind the leading edge (Linder et al., 1999), indicating

the importance of WASP in regulating cell polarity. Podosomes are also normally present in osteoclasts, where are required for bone resorption (Biswas et al., 2004). WASP-deficient osteoclasts from WASP knock-out mice displayed a reduction in the number of podosomes formed upon adhesion to bone slices, and although they could form actin plaques, these structures displayed abnormal organization compared with the actin rings formed by normal osteoclasts (Calle et al., 2004). These alterations led to impaired formation of sealing zones between cell membrane and bone and, consequently, to defective bone resorption. Although no evident bone alterations have been reported neither in WAS patients or in WASP knock-out mice, there is one report of a Caffey-like disease with the clinical features of infantile cortical hyperostosis observed in three WAS patients (Abinun et al., 1988). Similarly to what observed in WASP-deficient macrophages and osteoclasts, WASP-deficient immature dendritic cells (DC) were also found to lack podosomes, to display an altered morphology and to be unable to establish cell polarity and translocate in response to fibronectin (Binks et al., 1998; Burns et al., 2001). Moreover, immature DC from WAS patients had a reduced ability to migrate in response to MIP-1 α , a ligand for the receptors CCR1 and CCR5 (Allavena et al., 2001), while the migratory capacity of mature DC from WAS patients in response to MIP-3 β /CCL19 was normal. MIP-3 β /CCL19 is one of the ligands (together with SLC/CCL21) of the receptor CCR7, whose expression is induced in DC upon maturation (Allavena et al., 2000). Similar defects of motility resulted in a reduction of net translocation of WASP-deficient murine immature DC on fibronectin, as well as of mature DC in response to CCL21 (de Noronha et al., 2004). The relevance of these findings was highlighted in the same work by showing defective homing of DC from the periphery to local lymph nodes (LN), especially early after skin sensitization, and reduced ability to redistribute to T cell areas in the LN upon antigen challenge (de Noronha et al., 2004). Since podosomal structures disappear 2-4 hours after DC

maturation (Burns et al., 2004b) suggesting that they are important for the first phases of DC migration to secondary lymphoid organs, it is possible that the absence of podosomes in WASP-deficient DC contributes to the trafficking defects observed in vivo. The contribution of these DC abnormalities to the clinical phenotype of WAS remains to be determined, however, it is possible that poor T cell priming by DC occurs in the LN during infections. Moreover, the persistence of mature DC in the periphery could in part contribute to the initiation or maintenance of inflammatory processes.

In addition to the reported defects in cellular trafficking, an impairment in IgG-mediated phagocytosis by WASP-deficient human and murine monocytes/macrophages was reported (Leverrier et al., 2001; Lorenzi et al., 2000). Phagocytic process is involved in the removal of foreign material (usually derived from extra cellular pathogens), and is mediated by the binding of opsonizing immunoglobulins and complement components to FcR (Allen and Aderem, 1996). Ligation of FC γ R in macrophages initiates an intracellular signalling cascade with the recruitment and activation of several signalling molecules (Syk, PI3K, PLC) and adaptors (SLP-76, Nck). This process leads to the formation of a multi-molecular complex containing WASP, Fyb/SLAP, SLP-76, VASP and Nck, which localizes at the level of the phagocytic cup (Coppolino et al., 2001) and links FC γ R engagement to actin cytoskeleton reorganization. Thus, the absence of WASP is likely to impair the reorganization of actin and of the plasma membrane during phagocytosis. In addition, the phagocytosis and clearance of apoptotic cells by WASP-deficient murine macrophages was also shown to be defective (Leverrier et al., 2001). Clearance of apoptotic cells can be either mediated by direct recognition of PS exposed on apoptotic cells (Fadok et al., 2000) or by the binding of the complement component C1q, on the surface of apoptotic cells (Korb and Ahearn, 1997) and results in the production of anti-inflammatory cytokines, such as TGF- β . It is therefore possible that in WAS patients

impaired clearance of apoptotic cells contributes to disrupt immunological tolerance.

In accordance with data obtained in macrophages, it has been shown that WASP-deficient murine immature DC were impaired in the ability to process particulated Ag, probably as a result of impaired cytoskeletal function, while they were able to process and present protein Ags (Westerberg et al., 2003).

WASP was shown to play a role also in the signalling downstream the FcεRI (IgE receptor) in mast cells from WASP knock-out mice (Pivniouk et al., 2003). In these cells, both degranulation and production of IL-6 and TNF-α in response to FcεRI triggering were reduced. These functional defects seemed to be the consequences of reduced Ca²⁺ flux and JNKs phosphorylation, respectively.

Therefore, the absence of WASP affects a variety of cellular functions in the myeloid compartment. Overall, the processes of Ag presentation and of myeloid cell migration are likely to be impaired by WASP deficiency.

1.5.3 Defects in lymphoid cells

1.5.3.1 T cells

T cell dysfunction is generally considered the major cause of immunodeficiency in WAS patients (Burns et al., 2004a). My PhD project has focused on the study of different subsets of T cells, using either untransformed, IL-2-dependent, T cell lines or, in some cases, freshly isolated T cells. This introduction presents the knowledge in the field at the beginning of this project and the advancements, which have been made in these years, which, together with our contribution, helped to clarify the cellular and molecular basis of WAS pathology.

1.5.3.2 T cell activation

Early studies performed on T cell lines from WAS patients showed defective proliferation in response to anti-CD3 mAb, but normal proliferation in response to allogenic cells or IL-2 (Molina et al., 1992; Molina et al., 1993). Defective proliferation was found to be associated to the inability to secrete IL-2 in response to anti-CD3 mAb stimulation, although the addition of exogenous IL-2 was not able to rescue completely the proliferation. In that setting, co-stimulation through CD28 engagement was not able to elicit any increase of proliferation in WAS patients' T cells (Molina et al., 1993). In accordance with those data, which suggest that the absence of WASP impairs T cell activation induced by the T cell receptor (TCR), and possibly by CD28, the Ab response against a T cell-dependent protein Ag was found to be reduced in WAS patients (Ochs et al., 1980). Alterations of T cell homeostasis was reported in WAS, since reduced numbers of T cells in the blood were found in young patients, early after birth (Park et al., 2004). Interestingly, this reduction affected more profoundly naïve T cells than memory T cells and more profoundly CD8⁺ than CD4⁺ T cells. Possible explanations for these findings could be reduced thymic output or reduced survival/homeostatic proliferation in the periphery.

Two WASP knock-out mouse models were generated on different genetic backgrounds. In one model the cell maturation in lymphoid organs was not affected (Snapper et al., 1998) while in the other WASP deficiency caused a partial block of transition from double negative (DN) to double positive (DP) T cells in the thymus (Zhang et al., 1999). However, in both cases the numbers of circulating lymphocytes were lower in WASP-knock-out than in wild type (wt) animals (Dupre et al., 2006; Snapper et al., 1998). These data support a role of WASP in the survival of mature T and B cells. TCR-mediated T cell activation was found to be defective in WASP knock-out mice, both in terms of proliferation and IL-2 production. These defects were not

corrected by the addition of anti-CD28 mAb, suggesting that WASP is required for signalling through the TCR and, possibly, through CD28. Interestingly, the phenotype of WIP knock-out mice resembles WASP knock-out mice, showing impairment of T cell functions, including proliferation and IL-2 secretion (Anton et al., 2002), confirming the importance of WASP-WIP interaction for the regulation of WASP activity.

1.5.3.3 Cytokine production

At the beginning of this project, IL-2 production was basically the only defect in cytokine production attributed to WASP-deficient T cells. A more in-depth analysis of cytokine production has been performed during the development of this project, and will be presented and discussed hereafter. In addition, during these years a number of studies have been done in T cells from the mouse models of WASP, which have also highlighted a more complex picture of cytokine deficiency. Analysis of the basis of defective IL-2 production was performed in T cells from WASP knock-out mice, stimulated through TCR/CD28, showing that WASP absence determined a block in IL-2 gene transcription (Cianferoni et al., 2005; Morales-Tirado et al., 2004). This was the case both when freshly isolated total T cells or *in vitro* primed CD4⁺ T cells were analyzed, suggesting that WASP could be required for IL-2 gene transcription both in naïve and in memory T cells. IFN- γ secretion was also reduced in murine WASP knock-out T cells cultured in Th1 polarizing conditions and restimulated through TCR/CD28, although this reduction was shown to be due to defective cytokine granules sorting and secretion rather than to cytokine synthesis. Indeed, TCR/CD28-driven expression of IFN- γ mRNA and protein was normal in Th1 cells from WASP knock-out mice (Morales-Tirado et al., 2004). In the same work IL-4 production by WASP-deficient CD4⁺ T cells was also reduced.

1.5.3.4 CD8⁺ T cell activation and effector functions

At the beginning of this project, no data were available about the function of WASP-deficient CD8⁺ T cells. Recently, the contribution of CD8⁺ T cells to the immunological defects in the context of WASP deficiency was highlighted by the findings of a reduced secondary immune response against Influenza virus in WASP knock-out mice, which was due to a reduction in the frequency of Ag-specific CD8⁺ T cells, associated to defective synthesis of the effector cytokines IFN- γ and TNF- α by these cells (Strom et al., 2003a). The nature of this defect was next clarified, since it was shown that although WASP knock-out mice cleared normally Influenza virus during the primary inoculation, the frequency of memory Influenza-specific CD8⁺ T cells in the blood of infected WASP knock-out mice was lower than in wt mice (Andreansky et al., 2005). This suggests that WASP deficiency causes either reduced differentiation or survival of memory T cells. In addition, WASP knock-out mice showed increased susceptibility to lethal pneumonia induced by *S. Pneumoniae*, which was probably due to reduced production of natural Abs. Furthermore, these mice showed reduced clearance of *Mycobacterium bovis*. The clearance of this intracellular pathogen required coordinate action of macrophages and Th1 cells (Murray, 1999). However, macrophages from WASP knock-out mice secreted normal levels of both TNF- α , IL-12 and IL-10 upon *M. Bovis* exposure, suggesting that Th1 cell dysfunction rather than macrophages dysfunction is likely to be the cause of defective response to this pathogen. These data recapitulate some features of the immunological defects of WAS patient and suggest that WAS is characterized by defective cellular-mediated immunity.

1.5.3.5 Role of WASP in immunological synapse (IS) assembly

An important part of this PhD project has been to elucidate the molecular mechanisms by which WASP controls T cell activation. In the recent years a number of evidences underscored the importance of a structure, named the immunological synapse (IS), as a central regulator of T cell signalling and activation, and, more in general of information transfer between T cells and antigen presenting cells (APC). IS organization accompanies receptor clustering, together with recruitment of second messengers and adaptor molecules. Upon TCR engagement, these molecules re-organize according to a precise spatial distribution, with the TCR and associated src-kinases in the center, surrounded by a ring of integrins and cytoskeleton-associated molecules. This highly-ordered organization of signalling molecules has been called supra-molecular activation cluster (SMAC) and can be divided into a central SMAC (c-SMAC) and a peripheral SMAC (p-SMAC) according to the topology and molecule segregation (Monks et al., 1998). This structure has been named immunological synapse, for its analogies with the synapses formed by neuronal cells, and was shown to be required for sustained signalling and productive T cell activation. Indeed, at least 2 hours of IS contact are required to drive cellular division in a naïve CD4⁺ T cell (Lee et al., 2002). However, the role of the IS is clearly more complex than what initially proposed. The IS was shown to be dispensable for early tyrosine kinases signalling, upon TCR engagement, but required for TCR internalization and dampening of the signal (Lee et al., 2003; Lee et al., 2002). TCR desensitization and internalization is crucial to allow serial engagement of multiple TCR molecules by few peptide-MHC complexes (Valitutti et al., 1995b). Thus, the IS is probably involved in the modulation of the quantity and the quality of signals delivered through the TCR, so to determine the final outcome of T cell activation.

The formation of conjugates between T cell and APC implies changes in cell shape with massive re-orientation and re-organization of the cytoskeleton, requiring Ca^{2+} mobilization and actin polymerization (Donnadieu et al., 1994; Lowin-Kropf et al., 1998). Accordingly, the transport of the engaged TCRs from all over the cell membrane to the interface requires cytoskeletal transport via the activity of myosin/actin (Wülfing and Davis, 1998) and the integrity of actin cytoskeleton was shown to be essential for productive T cell activation (Valitutti et al., 1995a). A key transducer of signals from the TCR to the actin cytoskeleton is Vav-1, a guanosine exchange factor (GEF) for Rho GTPases including Rac1 and, likely, Cdc42, specifically expressed in T cells. Evidence of the importance of Vav-1 in the regulation of actin cytoskeleton, downstream of TCR triggering, came from the study of Vav-1 deficient murine T cells, which showed defective capping of actin and of TCR molecules (Holsinger et al., 1998). Similarly, TCR capping and endocytosis, two processes known to require actin polymerization, were impaired in T cells from WASP knock-out mice (Snapper et al., 1998; Zhang et al., 1999). The similarities between Vav-1- and WASP-deficient T cells support the possibility that Vav-1 acts as a GEF for Cdc42, downstream TCR/CD28, and that this pathway links the TCR to actin polymerization, through the action of WASP. In the recent years, many important pieces of information have been collected regarding a possible involvement of WASP in IS organization. WASP was initially shown to be recruited at the site of contact between T cell and APC (Cannon et al., 2001). This recruitment is achieved by the formation of a multi-molecular complex, constituted by the Src homology 2 domain-containing leukocytes protein of 76 kDa (SLP-76), Vav-1, the SH3-containing adaptor protein Nck and Cdc42 (Zeng et al., 2003). While Cdc42 stimulates WASP activity, as previously said, Nck binding was shown to be required for the re-localization of WASP to the IS (Zeng et al., 2003). WASP sub-cellular localization is also regulated by WIP. Indeed, in resting T cells the vast majority of

WASP is bound to WIP. Activation of T cell through the TCR induces phosphorylation of WIP, probably exerted by the non-classical protein kinase C θ (PKC θ), and its dissociation from WASP, which is then activated (Sasahara et al., 2002). PKC θ , which is predominantly expressed in haematopoietic cells (Baier et al., 1993) has been identified in the last years as a central regulator of T cell activation. This kinase was shown to be involved in IL-2 gene transcription, by regulating the activation of different transcription factors, including AP-1, NF κ B and NFAT (see section 1.5.3.7) (Baier-Bitterlich et al., 1996; Coudronniere et al., 2000) and to be recruited at the IS upon TCR/CD28 triggering (Bi et al., 2001; Monks et al., 1998). The importance of WASP in IS formation was highlighted by studies performed in both human and murine WASP-deficient T cells clearly showing that WASP absence impaired both the polarization of T cells toward beads coated with anti-CD3/anti-CD28 mAbs (Dupre et al., 2002) and the formation of the IS, particularly in conditions of suboptimal TCR triggering (Badour et al., 2003; Cannon and Burkhardt, 2004).

1.5.3.6 Role of lipid rafts in T cell activation

A crucial aspect of IS organization is the spatial segregation of surface receptors. This segregation was proposed to be achieved through compartmentalization of plasma membrane domains (Dustin, 2002). The lipid bilayer of plasma membrane is not homogenous but contains different lipid species, which are asymmetrically distributed in the cytoplasmic and exoplasmic leaflet and also precisely organized in the lateral dimension (Kusumi and Sako, 1996). Membrane domains enriched in sphingolipids and cholesterol, in the outer leaflet of the plasma membrane, were originally identified in epithelial polarized cells, in which an apical and a basolateral membrane was physically and molecularly distinguishable (Brown and Rose, 1992). These micro-domains (which are less than 70 nm in diameter) were proposed to act in living cells as platform (rafts) where proteins are “attached”, thus participating to a variety of cellular processes,

including protein sorting and signal transduction (Simons and Ikonen, 1997). Thanks to their particular lipid composition, rafts have been proposed to constitute a “liquid-ordered” phase of the plasma membrane. Since lipid rafts are relatively insoluble into non-ionic detergents (such as Triton X-100), it has been proposed that they can be isolated from native membranes as detergent-resistant membranes (DRM) (London and Brown, 2000). The importance of rafts in T cell activation came from the evidences that CD3 ζ is recruited to DRM after its engagement by anti-CD3 mAb (Montixi et al., 1998; Xavier et al., 1998). This re-localization was shown to put the TCR into contact with CD4-Lck, which are also present in DRM, where major tyrosine phosphorylation occurs. Indeed, both LAT (linker for activation of T cells) and the Src kinase Lck constitutively localize to lipid rafts thanks to lipid modification (Resh, 1996; Zhang et al., 1998). In addition, recruitment of several key signalling molecules into DRM upon TCR/CD28 triggering was shown to be crucial both for their activation and activity (for review see (Miceli et al., 2001)). Interestingly, the dynamic partition of the highly glycosylated phosphatase CD45 into DRM or non-DRM fractions was shown to regulate its inhibitory *versus* activatory role in T cell activation (Edmonds and Ostergaard, 2002; Zhang et al., 2005). Stimulation of T cells by beads coated with anti-CD3/CD28 mAbs, as well as by real Ag-loaded APC, was shown to induce clustering of the ganglioside GM1 (a glycosphingolipid containing sialic acid), which is enriched in a subset of membrane rafts (Bi et al., 2001; Round et al., 2005; Viola et al., 1999) further supporting a model of T cell activation in which re-organization of signalling machinery is mediated by transient partition in, or exclusion from, lipid rafts. Actin cytoskeleton was proposed to play a role in regulating lipid rafts dynamics and in particular in the clustering of lipid rafts at the IS (Sanui et al., 2003; Valensin et al., 2002; Yoon et al., 2003).

A further confirmation of the importance of the lipid composition of the plasma membrane in TCR/CD28 signalling came from the observation that T cell stimulation induces an upregulation of GM1 on the surface of T cells, as well as an increase of total GM1 levels (Viola et al., 1999). GM1 relocalization to plasma membrane was shown to occur in parallel with translocation of Lck from intracellular compartments, leading to the hypothesis that higher levels of plasma membrane GM1 correlates with a more efficient signalling machinery (Tuosto et al., 2001). Therefore, the higher GM1 levels observed in activated *versus* resting T cells, as well as in memory *versus* naïve T cells, would reflect higher responsiveness upon TCR triggering. In accordance with this hypothesis, hyper-reactivity of T cells from patients affected by lupus erythematosus were linked to higher levels of the ganglioside GM1 in activated T cells (Krishnan et al., 2004), suggesting that alteration in lipid rafts composition/dynamics could be involved in alteration immune function and regulation in humans.

Given the relevance of these micro-domains in T cell activation we investigated i) whether TCR/CD28-mediated stimulation could influence the partition of WASP into DRM or non-DRM fractions and ii) if and to what extent WASP deficiency influences the levels of GM1 in resting and activated cells.

Recently, a number of studies challenged or lead to a re-evaluation of the possible role of lipid rafts in T cell signalling, or even of the methods used to study rafts and raft-association of proteins. Therefore, our results will be discussed in view of these recent data.

1.5.3.7 TCR signalling: an overview

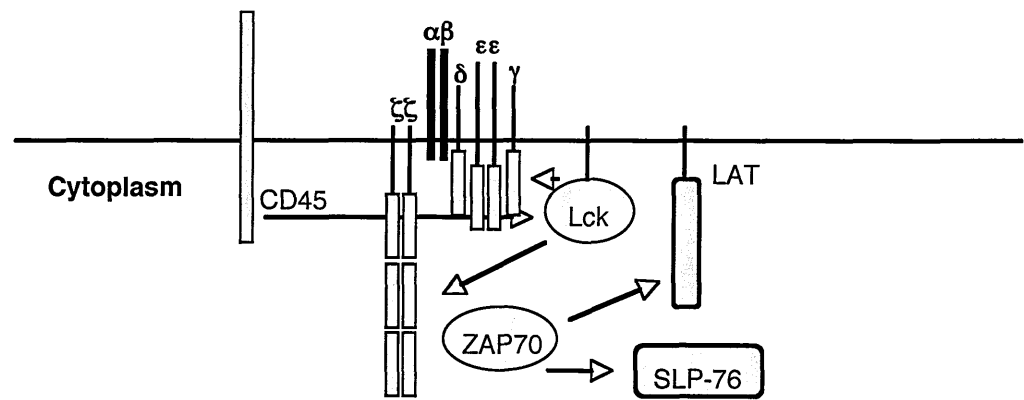
The detection of antigens by T cells is achieved through recognition by the TCR of antigenic peptides presented in association to MHC molecules. The final outcome of this recognition can be either full activation and acquisition of effector functions or establishment of anergy. The current model of T cell activation proposes that at least two signals must be provided to a naïve T cell to be fully activated and to avoid anergy (Linsley and Ledbetter, 1993; Schwartz, 2003). The first signal is necessarily delivered through the TCR, while the second signal, generally referred to as costimulation, is provided by receptors expressed by APCs or by soluble factors. The main molecule involved in the delivering of “signal two” is CD28, whose natural ligand (B7.1/2) is highly expressed by activated/mature APC (Acuto and Michel, 2003). Engagement of the TCR elicits a cascade of signalling events, which leads to transcriptional activation of multiple genes involved in T cell proliferation and differentiation, and to cytoskeletal rearrangements (van Leeuwen and Samelson, 1999). The main molecular events downstream of TCR and CD28 triggering will be presented here, in order to contextualise the “position” of WASP in these pathways, and to underline what is known about the role of WASP in TCR/CD28-mediated signalling and what is still unclear.

a. TCR signalling: proximal events

The first event upon TCR triggering is the phosphorylation of different tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3- γ , δ , ϵ and ζ invariant chains of the TCR. This phosphorylation is exerted by Src kinases (mainly Lck) and regulated by the phosphatase CD45 (Palacios and Weiss, 2004; Zamoyska et al., 2003). Phosphorylated ITAMs mediate the recruitment and activation of the Syk kinase ZAP-70 (Neumeister et al., 1995). Full phosphorylation of the CD3- ζ is essential for productive T cell activation (Sloan-Lancaster et al., 1994) and is

achieved with the help of CD4/CD8 co-receptors, which are constitutively associated to Lck. ZAP-70 is a central kinase as it phosphorylates a number of molecules including the adaptor molecules LAT (linker of activated T cells), which is constitutively rafts-associated (Zhang et al., 1998) and SLP-76. LAT-SLP-76 complex is a fundamental organizer of TCR signalling (Figure VA) (Myung et al., 2000).

Figure VA

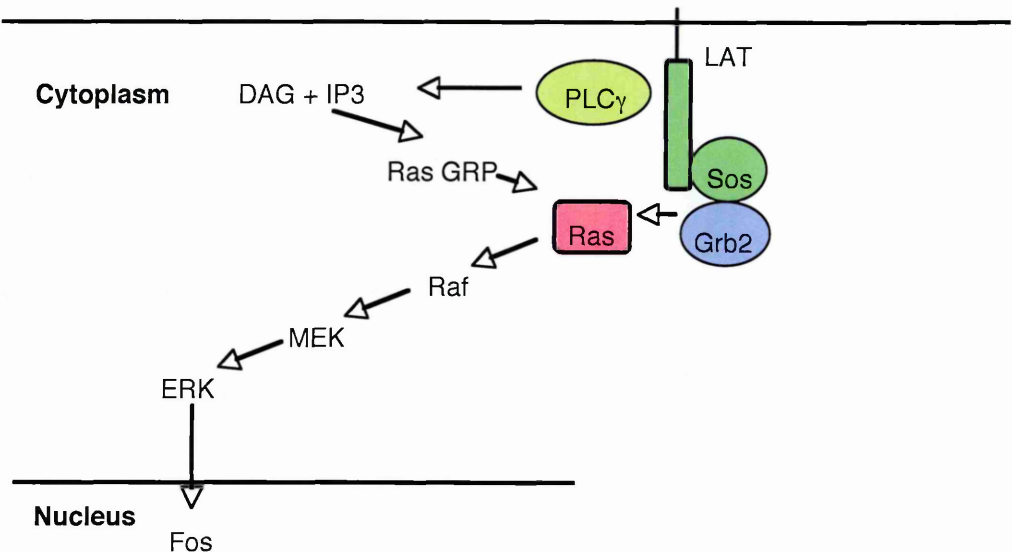


b. ERKs activation

One crucial function of LAT is coupling the TCR to the Ras pathway. Indeed, phosphorylated LAT recruits the adaptor protein Grb2, which associates with the Ras guanine nucleotide exchange (GEF) protein, Sos. Sos/Grb2 can activate the small G protein Ras, although an alternative pathway of Ras activation has also been described, involving the guanyl nucleotide releasing protein GRP, which can be recruited by DAG (see after) (Cantrell, 2003a). Activation of Ras is a crucial event, as it initiates the activation of the Raf-1/MEK/ERK1/2 signalling pathway. ERK1/2 (also called p44/42 MAPKs) controls T cell cycle progression and the transcription of the Fos gene (FigureVB) (Hunter and Karin, 1992). Fos gene transcription is essential for the

activation of the AP-1 complex, which is required for IL-2 gene transcription (see section g).

Figure VB



c. Inositol lipid metabolism (I): from PLC γ to activation of NFAT

In addition, LAT, together with SLP-76 and the adaptor Gads, mediates the recruitment of PLC γ , a key molecule of the inositol lipid metabolism. PLC γ activation requires the activity of Lck, Zap-70 and of the Tec kinases (see section e). The products of PLC γ enzymatic activity are inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 is responsible for release of Ca²⁺ from the endoplasmic reticulum (ER), which then mediate the opening of Ca²⁺ channels (Ca²⁺ release activated channels: CRAC) on the plasma membrane (Lewis, 2001). Ca²⁺ increase induces the activation of several calmodulin-dependent enzymes, including calcineurin and CAMKII. Calcineurin has a crucial role as it dephosphorylates multiple phosphoserine residues on the transcription factors NFAT. Phosphorylated NFAT is retained in the cytoplasm in resting cells, and upon dephosphorylation it translocates to the nucleus, where it

mediates the activation of many cytokine genes, including IL-2, IL-4, IFN- γ , IL-3, GM-CSF, IL-10 (Hogan et al., 2003). Five NFAT family members have been identified, which are named NFAT-1-2-3-4 (also called NFATc2-1-4-3) and NFAT-5 (also called TonEBP), which is regulated in a different way. The different members of NFAT family have a quite redundant role in T cell activation, as shown by the analysis of mice lacking individual NFAT proteins (Crabtree and Olson, 2002). However, NFAT2 alone is sufficient and necessary for Th2 differentiation (Yoshida et al., 1998).

d. Inositol lipid metabolism (II): from PLC γ to activation of PKC

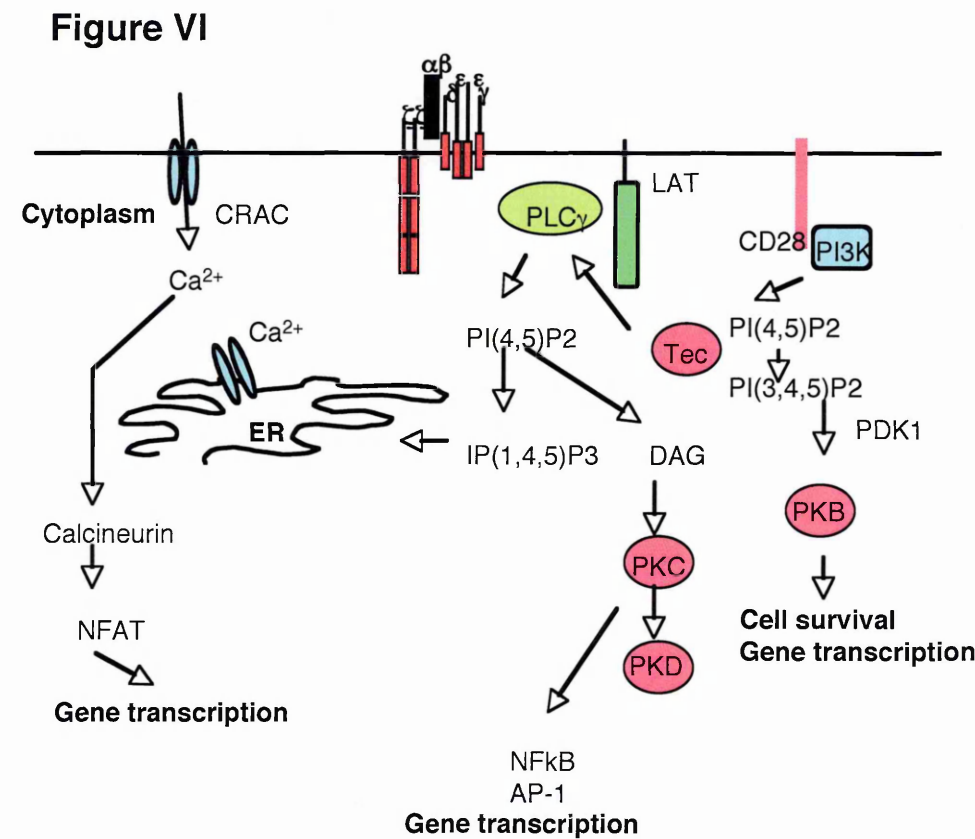
Production of DAG is critical for the activation of the serine/threonine kinases of the PKC family and also to link lipid metabolism to the Ras pathway (Ebinu et al., 2000). PKCs can be divided into two groups according to their dependence on Ca²⁺ (Mellor and Parker, 1998). In T cells the more relevant form of PKC is the Ca²⁺-independent PKC θ . The importance of PKC θ in T cell activation has been highlighted in the last years. This kinase is specifically expressed in T cells (and in skeletal muscle). Upon TCR triggering, PKC θ translocates to the IS at the levels of lipid rafts (Bi et al., 2001) and it positively regulates IL-2 gene transcription, likely by modulating the activity of the transcription factors AP-1 and NF κ B (Bi et al., 2001; Lin et al., 2000; Werlen et al., 1998).

e. Inositol lipid metabolism (III): PI3K signalling

A second pathway of inositol lipid metabolism activated by TCR and CD28 involves the activation of PI3K (Cantrell, 2001). PI3K can be divided into three classes. Class I PI3Ks are generally coupled to extracellular stimuli. In T cells, PI3K, which can be also activated through CD28, independently on TCR triggering (Ward et al., 1993), generates PI(3,4,5)P3, starting from PI(4,5)P2. PI(3,4,5)P3 is then metabolized to produce PI(3,4)P2. Generation of PIP3 and PI(3,4)P2 is crucial as these lipids mediate the recruitment of several proteins containing pleckstrin homology (PH) domains,

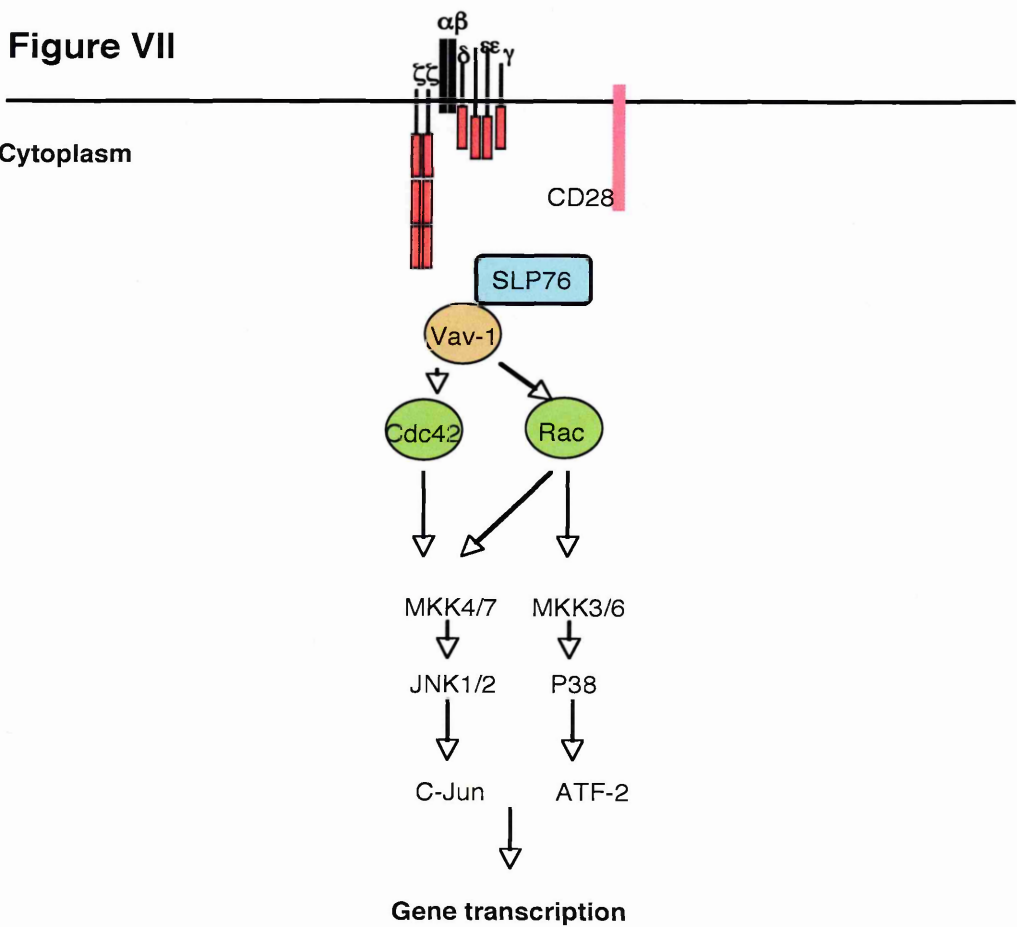
including the Tec kinases (Finkelstein and Schwartzberg, 2004), the phosphoinositide dependent protein kinase (PDK) 1, the protein kinase B (PKB/AKT) (Cantrell, 2001) and, possibly, Vav-1, which is the guanine nucleotide exchange proteins (GEFs) for Rho, Rac and Cdc42 . PDK1 targets different kinases, including p70SK1, GSK3 and PKB itself, thus regulating a variety of processes including T cell size, growth, survival, glucose metabolism and NFAT localization (Cantrell, 2003b; Patra et al., 2004).

Tec kinases belong to a large family of non-receptor tyrosine kinases. T cells mainly express Itk and Rtk Tec kinases (Schwartzberg et al., 2005). Beside being required for full PLC γ activation, Tec kinases exert an important role in coupling TCR/CD28 triggering to actin cytoskeleton rearrangement, by association and functional interaction with Vav-1 and WASP. A schematic representation of inositol lipid metabolism is depicted in Figure VI.



f. Activation of JNK and p38 MAPKs

Activation of Rho GTPases links TCR/CD28 triggering to the activation of the MAPKs JNK1/2 and p38 (Figure VII) (Davis, 2000; Dong et al., 2002). Both p38 and JNK are involved in the transcriptional activation of IL-2 gene, the first by phosphorylation of ATF2 transcription factor, the second by phosphorylation of the c-Jun transcription factor, which, together with Fos, forms the AP-1 complex (Figure VII) (Macian et al., 2001). In addition, JNK has been shown to stabilize IL-2 mRNA (Chen et al., 2000).

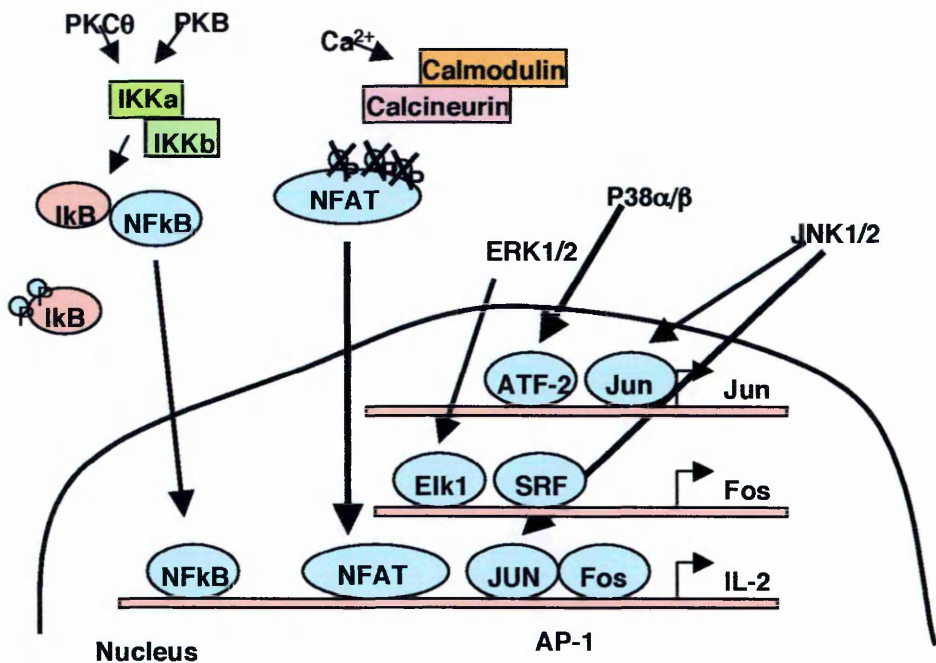


g. AP-1 and NFκB

AP-1 complex is formed by Fos family members (c-Fos, FosB, Fra-1 and Fra-2) and by Jun family members (c-Jun, JunB and JunD). AP-1 dimer binds DNA either alone, or together with NFAT (Karin et al., 1997). During T cell activation, AP-1 binds and positively regulates the IL-2 promoter. NFκB comprises a family of factors which acts as dimers. NFκB is retained in the cytoplasm of resting T cells through the binding to the inhibitors of κB (IκBs). Phosphorylation of IκBs by IκB kinases (IKKs) determines their degradation through the ubiquitin-proteasome pathway. PKCθ is involved in NFκB activation, possibly by direct phosphorylation of IKKβ (Lin et al., 2000). It has been recently proposed that PKCθ regulates NFκB activation through interaction with the CARMA1-Bcl-10-MALT1 complex (Thome, 2004). Interestingly, this complex is localized at the site of IS formation, at the level of lipid rafts (Wang et al., 2004). Therefore, formation of the IS could be required for the productive interaction of PKCθ with its downstream targets and eventually NFκB activation. Importantly, NFκB activity is also regulated by direct phosphorylation of its transactivating subunits p65, c-Rel and relB (Weil and Israel, 2004). NFκB dimers bind to IL-2 promoter, contributing to IL-2 gene transcription.

A schematic representation of IL-2 gene transcription is depicted in Figure VIII.

Figure VIII



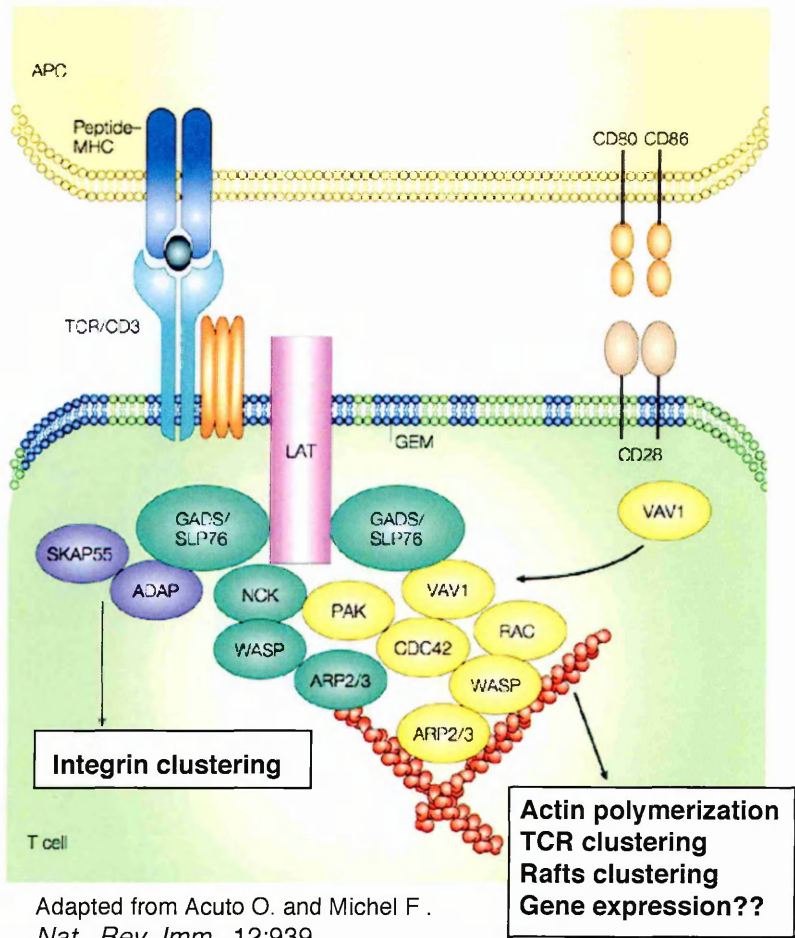
h. TCR/CD28 and the actin cytoskeleton: a central role for Vav-1 and WASP

Recruitment and activation of Vav-1 is another key event of TCR/CD28-mediated re-organization of the actin cytoskeleton. Vav-1 acts as a GEF for Rac and possibly for Cdc42, although this issue is more controversial (Tybulewicz, 2005).

In view of the fact that Vav-1 is activated through the TCR/CD28 and it induces the activation of Rho GTPases, Vav-1 has been recognized as a crucial transmitter of signals from the plasma membrane to the cytoskeleton and a crucial molecule, which allows to integrate signals from the TCR and from CD28. Vav-1 is recruited and activated through the binding to SLP-76 upon TCR triggering (Tuosto et al., 1996), while it possible that a different mechanisms account for Vav-1 recruitment upon CD28 triggering (Klasen et al., 1998; Michel and Acuto, 2002; Raab et al., 2001). Activated Vav-1 is found in a complex containing the adaptor proteins SLP-76 and Nck, and WASP (Zeng et al., 2003). Through its GEF activity, Vav-1 has been shown to regulate

actin cytoskeleton remodelling during IS formation and T cell activation (Wulfing et al., 2000). Downstream effectors of Vav-1 activity include WASP. WASP is therefore a key molecule involved in the transduction of signals from triggered TCR/CD28 to actin (Figure IX). Vav-1 has a crucial role not only in cytoskeleton rearrangement, but it was also shown to activate the Ras pathway and to regulate PKC θ localization and activation (Caloca et al., 2003; Villalba et al., 2000).

Figure IX



Adapted from Acuto O. and Michel F .
Nat. Rev. Imm., 12:939.

Whether CD28 provide just a quantitative or also a qualitative contribution to TCR signalling is still a matter of debate. In accordance with the first model, triggering of CD28 alone leads to transient expression of few genes, while TCR triggering is required for activation of the main transcriptional programs (Acuto and Michel, 2003). Therefore, CD28 would act as an amplifier of TCR signalling, being required to lower the TCR signalling threshold. The qualitative model would predict that CD28 can provide *in trans* help to TCR signalling, by activating distinct signalling pathways, possibly linked to cytoskeleton rearrangement and microdomains clustering. It is however possible that both mechanisms account for the essential contribution of CD28 to TCR signalling. Indeed, CD28 ligation was shown to both influence the phosphorylation status of different molecules, which are downstream of the TCR, and to enhance the phosphorylation of Vav-1 and WASP, both in the presence or absence of TCR engagement (Kim and White, 2006). Although this last study was performed in transformed T cells, it strongly suggests that Vav-1 and WASP are crucial molecules for the integration of TCR and CD28 signalling, which could determine the outcome of T cell activation.

1.5.3.8 TCR signalling in WASP-deficient T cells

Analysis of signal transduction pathways downstream the TCR in WASP-deficient human and murine T cells have unravelled some important aspects of the role of WASP in T cell activation. However, the precise mechanism(s) by which WASP regulates T cell activation remains to be determined. In particular, the biochemical link between structural events (such as IS formation), which directly require cytoskeleton remodelling, and physiological events (such as proliferation and cytokine production), which rely on transcriptional activation, is unclear.

Early studies showed that Ca^{2+} influx and CD3 ζ phosphorylation were elicited by TCR triggering in human WAS and control T cells at comparable levels (Molina et al., 1993). More in-depth analysis of TCR signalling was then performed in T cells from WASP knock-out mice, showing grossly normal activation of the main signalling events, including total tyrosine phosphorylation, and in particular phosphorylation of CD3 ζ and of the MAPKs ERK1/2 and SAPK/JNK, both in mature T cells and in thymocytes (Zhang et al., 1999). However, in this setting, TCR-driven Ca^{2+} influx seemed less sustained in WASP-deficient T cells than in control T cells, suggesting that despite early TCR signalling being conserved, downstream events, such as activation of transcription factors, could be disturbed. In accordance, a less sustained Ca^{2+} influx was observed in Vav-1 null Jurkat cells, which was associated with reduced transactivating activity of NFAT (Cao et al., 2002). Interestingly, this reduction was observed using both a consensus sequence extrapolated from the human IFN- γ promoter or from the IL-2 promoter. In the first case, productive activation required the binding of NFAT alone, while in the second case cooperative binding of NFAT and AP-1 was required. Moreover, in these cells defective induction of the SAPK/JNK pathway was reported, leading to reduced activity of AP-1. This was in accordance with previous data showing that activation of JNK/c-JUN/AP-1 pathways occurs downstream of Rac-1 and Cdc42 (Coso et al., 1995; Minden et al., 1995). Whether WASP is also involved in these pathways has been in part addressed in this work and will be discussed hereafter. In parallel to our work, studies performed in T cells from WASP knock-out mice support a role of WASP in the regulation of transcriptional activation of the IL-2 gene. Indeed, WASP was shown to regulate the activation of NFAT in thymocytes from WASP knock-out mice, stimulated through TCR/CD28 (Badour et al., 2004a). Moreover, nuclear levels of NFAT-2 (also called NFAT-c) were lower in CD4 $^{+}$ T cells from WASP knock-out mice than in wt mice, upon TCR/CD28 stimulation (Cannon and Burkhardt, 2004).

Defective NFAT-1 (also called NFAT-p) activity has also been recently reported in T cells from WASP knock-out mice. (Cianferoni et al., 2005). This defect was shown to be due to defective NFAT-1 dephosphorylation. Important pieces of information regarding the molecular mechanisms by which WASP regulates T cell activation came from the finding that, upon TCR triggering, WASP is phosphorylated at the level of tyrosine 291 by the Src-kinase Fyn, independently of Cdc42 activation (Badour et al., 2004a). This phosphorylation occurs early after TCR triggering and is transient. Dephosphorylation is exerted by the tyrosine phosphatase PTP-PEST, through the adaptor protein PSTPIP1. In WASP knock-out thymocytes, expression, of a WASP mutant in which the tyrosine residue 291 was mutated to phenylalanine impaired NFAT activation in response to TCR/CD28 triggering. Interestingly, the extent of this reduction was even bigger than the one observed in WASP knock-out thymocytes. In addition, actin polymerization and the accumulation of PKC θ at the site of IS formation were also impaired by mutation of the 291 tyrosine residue (Badour et al., 2004a).

WASP was also reported to play a role in TCR endocytosis. Indeed, defective TCR down-regulation, after TCR triggering, was reported in T cells from WASP knock-out mice (Zhang et al., 1999). This could be due either to a requirement of WASP in TCR endocytosis, mediated by the binding to the endocytic adaptor protein Intersectin2 (McGavin et al., 2001) or even to involvement of WASP in endosomes traffic, as it has been shown for the yeast WASP homologue, Las17 [Chang JCB 2005]. TCR internalization, which occurs rapidly after its engagement, is crucial to define the outcome of T cell activation. Internalized TCR can be either recycled back to the plasma membrane, where they can be engaged again (Valitutti et al., 1995b; Viola and Lanzavecchia, 1996), or they can be targeted to lysosomal degradation. Therefore, altered TCR internalization could also affect TCR signalling in WASP-deficient T cells.

However, whether all the signalling defects identified in WAS T cells depend on the loss of WASP-driven actin polymerization or if WASP could play a role in propagating TCR signalling, which is independent of actin polymerization, is a matter of debate. Indeed, it was shown that the WH1 domain of WASP, which is responsible for WIP binding, is strictly required for the induction of NFAT activity (Silvin et al., 2001). In the same work, it was shown that transfection of Jurkat cells with a mutant of WASP lacking the C terminal domain led to enhancement of NFAT activity, although TCR capping and internalization were not affected. These data led to formulate the hypothesis that regulation of IL-2 transcription by WASP could be uncoupled from actin polymerization. Recent data obtained in T cells from WASP knock-mice showed that although ERK1/2 phosphorylation, upon TCR/CD28 stimulation, was normal, the nuclear translocation of ERK1/2 was reduced, in comparison with wt T cells (Cianferoni et al., 2005). This defect was associated to reduced induction of c-Fos gene transcription, which is dependent on ERK1/2 signalling, and reduced AP-1 DNA binding activity. Therefore, it appears that WASP deficiency results in alteration of multiple pathways leading to T cell activation. Whether these signalling defects are all consequent to alteration of actin dynamics remains to be clarified.

1.5.3.9 NK cells

The high susceptibility of WAS patients to viral infections and lymphoreticular malignancies suggests an impairment of immune-surveillance. Although the percentages of NK cells in the peripheral blood of WAS patients is normal or even higher than the normal range (Gismondi et al., 2004), both the natural and antibody-mediated cellular cytotoxicity (ADCC) of NK cells isolated from either WAS or XLT patients was shown to be reduced with respect to NK cells of healthy donors (Gismondi et al., 2004; Orange et al., 2002). Reduced lytic activity was attributed to alteration of actin dynamics in WASP-deficient NK cells. Indeed, it was shown that NK-mediated killing requires the

formation of a highly organized structure, named activatory immunological synapse (IS), which is accompanied by re-localization of signalling proteins and lipid rafts (Lou et al., 2000; Vyas et al., 2001). WASP was shown to be recruited to the IS in NK cells (Orange et al., 2002) and its absence resulted in reduced clustering of F-actin and perforin-containing granules and in a general alteration of the spatial and temporal organization of this structure (Gismondi et al., 2004; Huang et al., 2005; Orange et al., 2002). Moreover, the formation of conjugates between NK and target cells itself was also shown to be impaired in the absence of WASP (Gismondi et al., 2004). Interestingly, culture in the presence of exogenous IL-2 was able to restore normal F-actin clustering and cytotoxicity in both WAS and XLT-derived NK cells (Gismondi et al., 2004; Huang et al., 2005), indicating that WASP is dispensable for IL-2-mediated signalling in NK cells. WASP is likely to regulate actin cytoskeleton reorganization during IS formation in NK cells, downstream of Cdc42, which is activated in response to the Fc γ RIII (CD16) triggering. Further information about the molecular mechanisms by which WASP controls NK cell activation and cytotoxicity came from the observation that WASP was tyrosine-phosphorylated in response to either CD16 or β 2 integrin engagement (Gismondi et al., 2004). In accordance to what observed in T cell from WASP knock-out mice, in which WASP phosphorylation was shown to be involved in the activation of the nuclear factor of activate cells (NFAT), in human NK cells, WASP absence resulted in delayed nuclear translocation of NFAT-2 and also of the RelA subunit of NF κ B (Huang et al., 2005). These alterations in transcription factors activation seemed to be actin-independent. This supports a role of WASP in modulating the transcriptional status of lymphoid cells, which does not depend on its role of actin nucleation-promoting agent, as it was previously shown in a different cellular model (Silvin et al., 2001). The importance of WASP not only in NK cell function but also in their survival has been pointed out by the finding of a patient with a severe WAS gene mutation in which a

somatic reverse mutation, likely occurred in a NK cell progenitor, led to the presence of a high proportion of WASP⁺ NK cells in the peripheral blood (Lutskiy et al., 2005), suggesting that WASP expression confers a selective advantage to NK cell progenitor. Moreover, since this high frequency of revertant cells has been found in the CD56^{dim} population, which comprises terminally differentiated NK cells, it is possible that WASP is more required for survival and/or function of terminally differentiated “effector” NK cells.

Therefore, impaired NK cell function could be one mechanisms underlying the high susceptibility to viral infections and to haematological tumours observed in WAS patients.

1.5.3.10 B cells

The role of WASP in Ab production and, more in general B cell function is still controversial. The first report of immune dysfunction in WAS patients was the inability to produce Abs directed against carbohydrate Ag (Ayoub et al., 1968), such as *Pneumococcus* polysaccharides. This family of Ags is defined as thymus-independent (T-I) as these Ags are in principle able to trigger B cell activation without the requirement for T cell help. Although polysaccharide Ags normally trigger the production of IgG2 Abs (Siber et al., 1980), normal serum distribution of IgG subclasses were reported in WAS patients, despite reduced levels of Abs directed against various polysaccharides Ags (Nahm et al., 1986), suggesting that other mechanisms could contribute to this deficiency. Reduced numbers of B cells were found in the blood of WAS patients (Park et al., 2005). This deficiency was associated to an increase in the frequency of CD21⁻ and CD35⁻ B cells, and, in patients older than three years of age, to a reduction in CD27⁺ post germinal centre B cells. CD21 and CD35 are both complement receptors, involved in phagocytosis of opsonized particles and in B cell response to T-dependent Ags (Ahearn et al., 1996). CD27, a member of

the TNFR family, is expressed by circulating memory B cells. In humans, about half of circulating memory B cell have undergone somatic hyper-mutation in the germinal centres (GC) (Klein et al., 1998), thus CD27 deficiency correlates with the involution of germinal centre (GC) observed in the spleen of WAS patients (Vermi et al., 1999). Importantly, CD27 is also expressed by marginal zone (MZ) B cells, which, in humans, are memory cells expressing high levels of CD21 (Pillai et al., 2005) and are mainly involved in the response against encapsulated bacteria, as they can produce anti-polysaccharides Abs. In WAS patients a depletion of the MZ was observed in the spleen (Vermi et al., 1999) could be one reason for the poor immune response to encapsulated bacteria. Similar alteration in the B cell areas of the spleen have been recently reported in WASP knock-out mice (Westerberg et al., 2005). The development of MZ B cells was shown to crucially depend, in the mouse, on signalling through Pyk-2 and NF- κ B1 (p50) (Cariappa et al., 2000; Guinamard et al., 2000). However, no data about the function of these proteins in WASP-deficient B cells are available. Contrasting data were obtained regarding transmembrane signalling in WASP-deficient B cells, showing either normal or reduced Ca^{2+} influx upon Ig crosslinking (Henriquez et al., 1994; Simon et al., 1992). A possible involvement of WASP in signalling through the B cell receptor (BCR), however, was supported by the finding that WASP was tyrosine-phosphorylated by the Bruton's tyrosine kinase Btk, upon BCR triggering (Baba et al., 1999), suggesting that WASP may be involved either in B cell development or activation. As expected, WASP is involved in cytoskeleton remodelling in B cells. Indeed, both in human and murine WASP-deficient B cells defective polarization in response to IL-4 and CD40 ligation and defective chemotaxis towards a variety of chemokines (CXCL13, CCL19, CXCL12) was reported (Westerberg et al., 2001; Westerberg et al., 2005). In the same work, reduced homing capacity of murine WASP-deficient B cells to both peripheral lymphoid tissues and to BM was found to be

associated to reduced immune response in WASP knock-out mice. Therefore, it is likely that defective trafficking of B cells contributes to WAS associated immune-deficiency.

It is still unclear what is the relative contribution of B versus T lymphocytes in WAS-associated immunodeficiency. Studies from WASP and WIP knock-out mice showed an overall preserved B cell function, concomitant with reduced T cell function, supporting the hypothesis the WAS immunodeficiency is mainly due to T cell rather than B cell dysfunction (Anton et al., 2002; Snapper et al., 1998). However, while in the WIP knock-out mice the immune response to a T-dependent Ag was compromised (Anton et al., 2002) this was not the case in the WASP knock-out mice (Snapper et al., 1998). It would be of interest to study Ab production by B cells isolated from WAS patients and in parallel evaluate the ability of T cells from WAS patients to stimulate Ab production by B cells.

1.5.4 Not only one synapse: a general defects in cell-cell cross-talk in WAS?

The term IS was initially used to refer to the interface formed by Ag-experienced T cells and Ag-pulsed B cells (Monks et al., 1998). A similar structure, i.e. a “mature” synapse, is also formed by naïve CD4⁺ T cells and CD8⁺ T cells with APC (Lee et al., 2002). These structures can be referred to as “stimulatory” synapses, as they sustain the activation of the involved T cells. However, synaptic-like structures can be formed also for other purposes: a typical example is represented by the conjugate formed between a CD8⁺ cytotoxic T lymphocyte (CTL) and its target cell. The notion that lytic granules polarize at the site of CTL-target cell contact was already known (Yannelli et al., 1986). However, more detailed studies showed that polarization of lytic granules occurs in a defined region, which is juxtaposed to the one where signalling molecules accumulates (Stinchcombe et al., 2001) (Figure IVB). In addition to polarized secretion, this IS allows also the transfer of membrane proteins from the target cell to the activated CTL (Stinchcombe et al., 2001). Acquisition of protein

determinants from target cells has been linked to fratricide killing and dampening of the CTLs response (Huang et al., 1999). In addition, as previously said, also NK cells form mature synapses in which a cSMAC and a pSMAC can be recognized, upon encounter with a susceptible target cell. Interestingly, formation of conjugates with non-permissive target cells seems to inhibit the passage from an immature to a mature synapse (Vyas et al., 2001).

The role of APC was not really much considered, in the early time of IS discovery, however, some studies indicate that APC are not just passive “observers” but that they actively polarize their cytoskeleton during contact with T cells (Al-Alwan et al., 2001). This finding is of particular interest in the context of WAS, as implies that the absence of WASP could in principle alter the ability of different haematopoietic cells to correctly interact with other cells of the immune system, or with cells outside the immune system. In accordance with this hypothesis, WASP was shown to be required for polarization of IL-12-containing granules in mature DC, a process crucial for the activation of NK cells (Borg et al., 2004).

In addition, synaptic-like structures were shown to form between T cells upon encounter of DC in the absence of the Ag (Revy et al., 2001). These interactions could be involved in naïve T cell survival. It is then possible that impaired conjugates formation between naïve T cells and DC contribute to the loss of naïve T cells reported in the blood of WAS patients (Park et al., 2004). Investigation of T cell-DC contacts in a intact lymph node would provide important clues about physiological T cells homeostasis in the context of WAS.

1.6 WAS and XLT: clinical management

1.6.1 Current treatments

Patients with classical WAS have a poor prognosis if untreated. As the risk of haemorrhages is one of the most frequent and severe complications of WAS, splenectomy is commonly recommended for WAS patients with serious bleeding problems and it was found to significantly improve platelets counts and reduce the risk of haemorrhages in WAS patients (Mullen et al., 1993b; Sullivan et al., 1994). The efficacy of splenectomy is due to the fact that the spleen is the major organ where platelets destruction takes place. However, one of the major concerns about splenectomy is that it increases the risk of fatal sepsis (accounting for 13-15% of mortality in splenectomized patients), which however can be reduced by the administration of antibiotic prophylaxis. Another limitation of splenectomy is represented by episodes of relapse of thrombocytopenia and bleeding episodes (idiopathic thrombocytopenic purpura) that was reported in splenectomized WAS patients, partially caused by the presence of platelets-associated IgG (Mullen et al., 1993b). Thus, splenectomy is not an effective treatment, although it increases life expectancy and quality in WAS patients (Mullen et al., 1993a). To reduce the risk of infections, in many centres WAS patients are treated with intravenous immunoglobulins (IVIG) and antibiotic prophylaxis, however it is not clear whether these treatments effectively reduce the incidence of life-threatening infections (Conley et al., 2003). The treatment of choice for autoimmune manifestations in WAS patients is constituted by steroids, which, alone or in association with cyclosporine have been shown to be efficacious (Dupuis-Girod et al., 2003). However, the only resolutive treatment for WAS is bone marrow transplantation (BMT). BMT is generally the first choice therapeutic option for WAS patients for which an HLA-identical sibling donor is available. In these cases the percentage of BMT success (in terms of long term survival)

was 80-100% (Filipovich et al., 2001a; Mullen et al., 1993a; Ozsahin et al., 1996). Long-term survival was shorter if BMT was performed from a non-HLA-identical related donor (28%, 30% and 52% of the patients receiving this transplant were alive after 5 years in the three independent studies cited before). However, it has to be considered that this type of transplant has been generally used in patients with severe ongoing clinical manifestations, which often required active immunosuppression (Ozsahin et al., 1996). Moreover, the requirement for T cell depletion of donor marrow (used to lower the incidence of graft-versus-host disease –GVHD-) can increase the risk of post-transplant EBV-associated lymphoproliferative disease and severe infectious complications (Filipovich et al., 2001a; Ozsahin et al., 1996). However, a high percentage of success was also reported in WAS patients transplanted with BM cells from matched unrelated donor (MUD) before 5 years of age (mean survival at 5 years post transplant: 84% (Filipovich et al., 2001b)).

The overall poor outcome of BMT in WAS patients and the high variability of the clinical phenotype associate to this syndrome points out the importance of identifying the appropriate treatment for each patient. Recently, the possibility of a gene therapy approach has been envisaged basing on promising pre-clinical results obtained both *in vitro* and *in vivo* (see section 1.6.3.3). A critical evaluation of the safety of this approach will determine whether gene therapy can be considered a valid alternative to BMT when an HLA-identical sibling donor is not available.

1.6.2 Perspectives for the treatment of WAS: gene therapy

1.6.2.1 Retroviral and Lentiviral vectors for gene therapy

Among the vectors currently available for gene therapy, retroviral vectors (RV) are the most widely used. Most of them, as the one used in this work, were derived from the retrovirus MMLV (Moloney murine leukemia virus) (reviewed in (Verma and Weitzman, 2005)). The genome of MMLV is constituted by a single strand RNA,

which, after infection of the host cell, is transcribed into a double strand DNA molecule, by the viral enzyme reverse-transcriptase (pol). Transport of the viral DNA to the nucleus requires nuclear membrane breakdown occurring during mitosis (Roe et al., 1993). Therefore, MMLV and MMLV-derived retroviral vectors are able to infect only dividing cells. The viral genome is constituted by three main genes: gag, pol and env, which encodes respectively for structural proteins (gag), reverse transcriptase and integrase (pol), and proteins of the viral envelope (env). The peculiarity of retrovirus, which make them suitable and safe vectors for gene therapy, is that the only elements required for viral replication and packaging *in cis* are represented by the 5' and 3' long terminal repeats (LTR), which contain promoter, poly-adenylation sequence and the packaging site (ψ). All the other proteins can be supplied *in trans*. Therefore, all the genes encoding for proteins necessary to produce infectious particles can be removed and replaced with a cDNA encoding for the gene of interest. Transcription of this cDNA can be driven either by viral LTR or by an internal promoter. Since it is often necessary to express two genes within the same retroviral vector (usually one is the gene of interest and the other is a selectable marker gene), different strategies have been exploited to this purpose. One possibility is to use the viral LTR to drive the expression of one gene and an internal exogenous promoter to drive the expression of the second gene. However, the presence of two promoters, in the context of the same vector, reduces cDNA expression. Therefore, alternative strategies have been developed, such as the use of a polycistronic message with the addition of an internal ribosome entry site (IRES) (Zitvogel et al., 1994). To produce RV, packaging cell lines have been developed. GP+Am12 (Freas-Lutz et al., 1994) is a third-generation packaging cell lines, generated by stably transfecting NIH 3T3 cell line with two different plasmids, encoding for gag-pol and env proteins, respectively. Vector production by the packaging cell line is achieved by stable or transient transfection with the vector

plasmid, derived from the vector backbone and containing the ψ sequence and the gene(s) of interest. Safety of this system is guaranteed by the fact that three independent events of recombination would be necessary to produce a replication competent virus. GP+Am12 is an amphotropic packaging cell lines, since it express an envelope, which allows the infections of both murine and human cells.

While retroviral vectors can infect only dividing cells, lentiviral vectors (LV) derived from HIV-1 are able to infect both dividing and non-dividing cells (Naldini et al., 1996). Third generation lentiviral vectors are constructed in a way to address the biosafety requirements, which imply the removal of all the proteins involved in AIDS pathogenesis, while all the other structural and enzymatic proteins are provided *in trans* in three different plasmids. These vectors were shown to efficiently transduce human BM-derived repopulating CD34⁺ cells after short *ex vivo* manipulation (Guenechea et al., 2000) making them a suitable tool for gene therapy trials in haematological diseases. Moreover, the construction of so-called self-inactivating (SIN) vectors, which contain a deletion in the viral LTR, (Miyoshi et al., 1998) significantly reduced the risk of recombination of the vector in transduced cells (Bukovsky et al., 1999). LV are able to transfer complex genetic elements, including regulatory elements and to provide high level and stable transgene expression, in different cell types, including human CD34⁺ progenitor cells (May et al., 2000; Miyoshi et al., 1999). Regulatory elements would allow regulated and tissue-specific expression of the therapeutic gene, avoiding ectopic expression and reducing the potential toxicity of the treatment.

Both RV and LV carry the risk of insertional mutagenesis. This risk has been generally evaluated as very low, basing on the probability of insertion in a given site of the host cell genome. Recently, the issue of insertional mutagenesis has dramatically come to the attention, due to the occurring of adverse events in one ongoing clinical trial based on RV (which will be discussed in the next section). Therefore, the safety of

RV is now being reconsidered. LV are likely to become the vectors of choice for the future gene therapy clinical trial, as they accomplish two important safety requirements: the usage of transcriptional regulatory elements and low genotoxicity due to the possible influence of the vector on the transcription of neighbouring genes (Montini et al., 2006).

1.6.2.2 Gene therapy of primary immunodeficiency: the paradigm of X-SCID and ADA-SCID

Gene therapy approaches have been shown to be efficacious in the treatment of two different severe combine immunodeficiencies (SCID). These diseases are different from WAS, as the prognosis of affected children is more severe. Indeed, in SCID patients, death generally occurs in the first two years of age, due to overwhelming infections, if not treated. This is due to the profound deficiencies of lymphocytes, being different subsets variably affected depending on the genetic defect (Buckley, 2004). BMT in SCID patients has been shown to have an overall rate of success of 77% and 65% in two different retrospective studies (Antoine et al., 2003; Buckley, 2004). The treatment of choice is BMT from HLA-identical sibling donor, with a rate of survival of more than 90%, while BMT from HLA-haploidentical related donor is affected by higher risk of morbidity and mortality. Crucial issues was also the age at transplant, as the rate of success was higher if children were transplanted during the first 3.5 months of life (Buckley, 2004). In view of the higher risks for patients who do not have a HLA-identical donor available, correction of autologous haematopoietic stem cells by gene transfer has been exploited as an alternative therapy. Up to now, gene therapy has been successfully used to treat two types of monogenic SCID: X-SCID and ADA-SCID. The first originates from the lack of the γ subunit of the IL-2 receptor, also called the common γ chain (Kovanen and Leonard, 2004). The second is due to the lack of adenosine deaminase, an ubiquitous enzyme involved in the purine metabolism, whose

absence causes an accumulation of toxic metabolites, which is particularly detrimental to lymphoid cells, but also non-lymphoid tissues (Blackburn et al., 1998). Gene therapy trials were started, based on the use of retroviral vectors derived from the Moloney murine leukaemia virus (MMLV) containing the cDNA of the therapeutic gene. BM-derived CD34⁺ cells from the SCID patients were transduced *ex vivo* with the retroviral vector and re-infused in the patients (Cavazzana-Calvo et al., 2000; Gaspar et al., 2004). The French gene therapy trial of X-SCID was successful in the majority of patients, which were fully immune-reconstituted and who showed long-term transgene expression. However, three patients developed leukaemia, approximately 2.5 years after gene therapy. In two of these patients, transformed T cells expressed vector-derived γ -chain and the oncogene LMO-2 (Hacein-Bey-Abina S et al., 2003) (agence française de sécurité sanitaire des produits de santé –AFSSAPS–). This gene is normally expressed in haematopoietic progenitors and its locus is involved in chromosomal translocation in cases of acute of T cell leukaemia (Rabbitts et al., 1999). Indeed, ectopic LMO-2 expression in transduced T cells was due to insertion of the RV in the 5' region of the gene, within a chromosomal common fragile site (Bester et al., 2006; Hacein-Bey-Abina S et al., 2003). In addition, there is increasing evidence that γ -chain expression, driven by RV, plays a cooperative role in the process of oncogenesis, possibly by influencing T cell differentiation (Dave et al., 2004; Woods et al., 2006) In contrast, no adverse events were reported in the ADA-SCID gene therapy clinical trial. Eight patients have been enrolled in this trial up to now, of which six have been followed up for more than six months. All patients are healthy and show partial to full immune reconstitution, with improved systemic detoxification (Aiuti et al., 2002) (and A.Aiuti, *Personal communication*).

However, the occurrence of leukaemia in the French trial raised urgently the issue of insertional mutagenesis caused by RV integration. Analysis of the integration

pattern of RV in both cell lines and non-human primate haematopoietic progenitor cells revealed a preferential integration close to transcription start sites of active genes, while LV preferentially target the entire transcriptional unit of active gene, without a preference for the transcriptional start region (Laufs et al., 2003; Mitchell et al., 2004; Schroeder et al., 2002; Wu et al., 2003). Therefore, the main risk associated with RV seems to be the activation of a potential oncogene, while the main risk of LV could be the disruption of a regulatory gene. However, different parameters will influence the transcriptional profile of transduced cells, including the stage of maturity and the *ex vivo* culture conditions. Ongoing studies are being performed in order to assess the safety of LV in a setting of HSC transduction and transplant.

1.6.2.3 Gene therapy approaches for WAS

In the last years, considerable efforts have been devoted to the development of a gene therapy approach for WAS. The ability of either RV or LV-based WAS gene transfer to correct cellular dysfunction has been evaluated in haematopoietic cell lines derived from WAS patients and in WASP knock-out mice. As exposed later, part of my project has been focused on the comparison of the efficacy of RV *versus* LV vectors-mediated WAS gene transfer to correct WAS T cells defects.

The first studies were performed in immortalized B cell lines from WAS patients, showing that expression of WASP, mediated by RV, was able to restore actin polymerization and to partially correct the abnormal pattern of glycosylation of membrane proteins, which was considered one of the typical hallmarks of WAS-associated cellular abnormalities (Candotti et al., 1999; Huang et al., 2000). However, given the crucial role of T cells in WAS pathology, the main challenge remained the correction of these cells. Restoration of WASP expression in primary T cells from WAS patients by a MSC (mouse stem cell virus)-based retroviral vector containing human WASP cDNA under the control of the viral LTR lead to correction of proliferative

response to immobilized anti-CD3 mAb and partial correction of IL-2 production (Strom et al., 2003a). However, no data were available regarding the ability of transduced T cells from WAS patients to respond to sub-optimal stimulation. Correction of this parameter could be of crucial importance, given the role of WASP in T cell activation (see discussion). Therefore this point has been addressed in our study. Retroviral vectors were also used to transfer murine WASP cDNA, under the control of CMV promoter, into HSC of WASP knock-out mice. WASP-transduced HSC were found to differentiate in mature T and B cells, when transplanted into RAG2 congenic recipients, and correction of the proliferative response of T cells to anti-CD3 mAb was achieved (Klein et al., 2003). This system was particularly challenging because WASP knock-out mice spontaneously developed colitis within the first 4 months of age (Snapper et al., 1998). Although the nature of this colitis was not fully elucidated, transfer of WASP knock-out HSC into RAG2^{-/-} mice lead to the development of colitis, which was less frequent and severe in RAG2^{-/-} mice receiving HSC transduced with the WASP-encoding RV. In addition, RV-mediated transduction of murine WASP-deficient HSC and transplantation into WASP knock-out recipients significantly improved the secondary immune response to Influenza virus, which is defective in these mice (Strom et al., 2003b), providing evidence that WASP expression can restore immune functions *in vivo*. In the same work selective advantage of WASP-expressing T and B cells over WASP-deficient cells (but not of neutrophils and monocytes) was reported. The presence of *in vivo* selective advantage will be crucial in the outcome of a gene therapy treatment for WAS in humans.

The limits of the studies described here before are that RV used are not clinically applicable as they contain murine WASP cDNA and GFP, as a reporter gene and that the transduction efficiency achieved was generally low. In addition, only correction of T cells was evaluated. The use of third generation, self-inactivating, HIV-

1-derived LV led to promising results (some of them will be presented and discussed later) both in human WAS mature T cells and in WASP knock-out mice HSC. The proof of principle of the efficacy of human WASP in correcting cellular and immune dysfunctions in WASP knock-out mice was achieved by the use of a SIN HIV-1 derived vector (Charrier et al., 2005). LV transduction was able to restore physiological WASP expression and to correct T cell proliferative defects and cytoskeletal abnormalities in BM-derived DC. Interestingly, correction of this latter phenotype required physiological WASP expression, while correction of T cell proliferation to anti-CD3 mAb was achieved also with sub-optimal WASP expression.

Another important issue is the regulation of WASP. The use of WASP autologous promoter to drive WASP expression in a LV was exploited and resulted in tissue-specific expression of WASP in human cell lines (Martin et al., 2005). WASP autologous promoter would in principle allow physiological and regulated WASP expression. In this setting, it is possible to envisage functional correction of the cells achieved with low numbers of integrated vector per cell. This will be an important point for future safety studies.

1.7 WAS-associated autoimmunity: a role for naturally occurring T regulatory (nTreg) cells?

As previously said, WAS patients often suffer from autoimmune manifestations. This observation could seem paradoxical as these patients have variable degree of immunodeficiency, which generally implies poor reactivity of their T cells, in particular, and of the immune system, in general. However, the association between immunodeficiency and autoimmunity is not unusual. Patients affected by various primary immunodeficiency, including chronic granulomatous disease (CGD), familial hemophagocytic lymphohistiocytosis (FHL), hyper-IgM syndrome, complement

deficiency, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), immune dysfunction-polyendocrinopathy-enteropathy X-lined (IPEX) and common variable immunodeficiency (CVID) (Arkwright et al., 2002) present various autoimmune manifestations, which, in some cases, can be lethal. Autoimmunity has generally been attributed to the inability to clear pathogens and to a subsequent exacerbated and sustained inflammation. This seems indeed to be the case for some of the diseases cited here before, and it is also a possible mechanism underlying autoimmunity in WAS patients. However, another possible cause of autoimmunity could be the loss of tolerance to self Ags.

Immunological tolerance is a crucial feature of the immune system, which ensures the discrimination between harmful and non-harmful Ags. This second category comprises self Ags, food Ags and environmental Ags. During T cell maturation, cells harbouring self-reactive TCR are generated. The vast majority of these cells are deleted in the thymus by a process called negative selection, which eliminates cells which binds with too high affinity to complexes formed by self-peptides and self MHC (Sha et al., 1988). Interestingly, there are specific mechanisms allowing the ectopic expression of tissue-specific Ags in the thymus, in order to delete the potentially reactive T cells. One gene involved in this process is the autoimmune-suppressor gene (AIRE), whose mutations are responsible for APECED (Anderson et al., 2005; Nagamine et al., 1997). However, negative selection is not able to eliminate all the potentially reactive T cells and some of them are found in the periphery as mature T cells, in healthy individuals. In the periphery different mechanisms have been shown to control the potential detrimental effect of these cells. One mechanism is the induction of anergy. Anergy is defined as a state of long-term T cell unresponsiveness, consequent to partial activation (Schwartz, 2003). Another mechanism is active suppression of autoreactive effector T cells by regulatory T cells (Treg). In the last years, specialized subpopulations of Treg

cells have been discovered and their mechanism of action has been extensively studied, although it is still not completely unravelled. Regulatory T cells type 1 (Tr1) (Groux et al., 1997) were characterized on the basis of their unique profile of cytokine secretion. These cells are anergic to TCR stimulation *in vitro*, and their suppressive activity is mediated by secretion of IL-10 and transforming growth factor β (TGF- β) (Roncarolo et al., 2001a). Differentiation of naïve CD4⁺ T cells into Tr1 cells has been shown to occur upon stimulation with immature “tolerogenic” DC (Jonuleit et al., 2000; Roncarolo et al., 2001b). It is then conceivable that Tr1 cells are involved in the maintenance of active peripheral tolerance toward foreign non-harmful Ags or self Ags. Since DC status is important in the generation of these cell subset, it is possible that in WAS patients reduced or delayed migratory capacity of mature DC to the draining lymphnodes could alter the generation or homeostasis of Tr1 cells.

Another subset of Treg cells is constituted by the CD4⁺/CD25⁺/FoxP3⁺ cell population. These cells are called naturally occurring T regulatory (nTreg) cells, as there are a number of evidence indicating that they are produced in the thymus, as a mature and functionally distinct population. Indeed, they are present in the thymus both in humans and in mice (Darrasse-Jeze et al., 2005; Fontenot et al., 2005a). NTreg cells can be isolated from the peripheral blood of healthy subjects, where they constitute 5% of CD4⁺ cells (slightly higher percentages are found in the mouse) (Bacchetta et al., 2005). The more typical hallmark of nTreg cells has been for a while considered the expression of high levels of CD25, which is the α -chain of the IL-2 receptor. As CD25 is normally upregulated upon T cell activation, it is conceivable that nTreg represents a population of pre-activated (or primed) cells. More recently, the importance of the forkhead transcription factor FoxP3 as a distinctive marker of nTreg cells came from the evidences that mutation in this gene cause a severe autoimmune manifestations both in mice (Brunkow et al., 2001) and in humans, where they cause IPEX (Bennett et al.,

2001). The requirement of murine and human nTreg cells for the FoxP3 expression seems to be different. Indeed, FoxP3 mutation completely abolish the presence of CD4⁺/CD25⁺ nTreg cells in the scurfy mouse, while in patients affected by IPEX nTreg cells are present, but their suppressive ability is reduced (Bacchetta et al., 2006). In addition, ectopic expression of FoxP3 in murine naive T cells led to the acquisition of suppressive functions (Hori et al., 2003), while this was not to be true for human CD4⁺ T cells, where ectopic expression of FoxP3 led to the acquisition of “anergic” features but not of suppressive capacity (Allan et al., 2005). Whether nTreg cells really represent a separate lineage from effector T cells, arising uniquely from the thymus, or whether generation of nTreg is possible also in the periphery is still a matter of investigation.

The presence of nTreg cells in the periphery is required to actively maintain self-tolerance, indeed transfer of CD25-depleted CD4⁺ T cells into athymic nude mice is sufficient to induce several autoimmune manifestations (Sakaguchi et al., 1995). The mechanism of action of nTreg cells has not been completely unravelled, however, a number of evidences indicate that they require cell-cell contact for their suppressive activity. In some cases TGF- β was shown to be involved in nTreg suppressive capacity (Levings et al., 2002) or even in their generation (Chen et al., 2003). In addition, a clear, non-redundant, role of IL-2 in the generation and function of nTreg was reported (Malek and Bayer, 2004).

Another big issue regards the repertoire and specificity of nTreg cells. Different studies showed that the nTreg cells have a quite high affinity for self peptides (not as much to be negatively selected) (Jordan et al., 2001; Kawahata et al., 2002). Therefore it is possible that their specificity imply a predominant role in the maintenance of tolerance to self Ags.

Reduced suppressive activity of nTreg cells was reported in different human diseases with an autoimmune base, including multiple sclerosis (MS) (Haas et al.,

2005), rheumatoid arthritis (Ehrenstein et al., 2004) and, recently, IPEX (Bacchetta et al., 2006). It is therefore possible that a dysfunction in the generation or function of nTreg cells constitute one of the basis of WAS-associated autoimmune manifestations.

Data obtained in our laboratory, revealed that despite normal proportion and phenotype of nTreg cells are present in the thymus and in the spleen of WASP knock-out mice, their *in vitro* suppressive activity is defective, if compared to the one nTreg cells isolated from wild type mice (Marangoni F., *Personal communication*). In addition, an independent study performed in a different strain of WASP knock-out mouse showed that the ability of WAS nTreg cells to control autoimmune colitis, induced by the adoptive transfer of effector cells into SCID mice, is defective (Snapper S., *Personal communication*). Therefore, part of this project was dedicated to the characterization of the phenotype and the function of nTreg in WAS patients.

2 SPECIFIC AIMS

Wiskott-Aldrich syndrome is a complex genetic disease, caused by mutation in the gene encoding for WASP, with a major involvement of the immune system. WAS patients are more susceptible to both infections and autoimmune disorders than healthy individuals. WASP is expressed in all the haematopoietic cells, where it acts to regulate actin polymerization in response to extracellular stimuli. Although WASP deficiency impairs several cellular functions, thereby affecting both innate and adaptive immune response, a central role of T cells has been demonstrated in the pathogenesis of WAS.

Despite its well-characterized role as actin cytoskeleton regulator, it was not clear how, and to what extent, WASP deficiency could affect T cell activation and effector functions. In addition, the complex picture of WAS clinical manifestations, which includes immunodeficiency and autoimmunity, suggests that a general deregulation of T cell function/homeostasis could occur in these patients. The study presented here was integrated in the more general goal of our Institute to establish, in the future, an efficacious and safe protocol of gene therapy for WAS.

We therefore investigated i) the cellular and molecular mechanisms of WASP-mediated T cell activation in T cell lines from WAS patients, ii) the effector functions of CD4⁺ Th cells and of CD8⁺ CTLs from WAS patients, iii) the phenotype and the function of nTreg cells isolated from WAS patients and iv) the use of RV and LV to restore WASP expression in T cell lines from WAS patients, in order to correct their functional defects.

3 MATERIALS AND METHODS

3.1 Patients and T cell lines

3.1.1 Clinical phenotype

Patients W1, W2, W3, X6, W8, W12, W13, which have been studied here, were previously described (Dupre et al., 2002; Jin et al., 2004; Notarangelo et al., 2002). Patient W1 was diagnosed as full blown WAS (clinical score 4, according to Zhu Q. et al. (Zhu et al., 1997)), on the basis of clinical phenotype and WAS gene mutation (eczema, thrombocytopenia, recurrent HSV infections, candidiasis, pneumonia). He was been often treated with antibiotic prophylaxis and IViG infusions and his clinical conditions are stable. Patient W2 was diagnosed as full WAS (score 5) based on clinical symptoms and molecular analysis of the WAS gene. At the age of six years, he underwent bone marrow transplant from matched unrelated donor. He died in the first year following BMT due to Cytomegalovirus infection. Patient W3 was diagnosed as XLT (score 2). He was thrombocytopenic and during infancy he developed severe eczema and haemorrhagic diathesis. Patient W4 was diagnosed as full-blown WAS (score 5), he developed mild eczema and autoimmune manifestations, including vasculitis, arthritis and IgA nephropaty. Patient X6 was diagnosed as XLT (disease score 0.5) and shows thrombocytopenia and transient eczema but he has not a history of infections. Patient W8 was diagnosed as full WAS, he had thrombocytopenia and mild eczema, with recurrent otitis media (disease score 3). He died of intracranial haemorrhage at the age of three years. Patient W11 was diagnosed as full blown WAS (score 5). At diagnosis he had bloody diarrhea and was severely thrombocytopenic and during the first two years of life he experienced autoimmune haemolytic anemia, which was treated with steroids administration, and presented severe eczema, dermatitis and

petecchiae. He underwent BMT from MUD at the age of 1 year. At the time of blood sampling he was under steroids treatment. Patient W12 was diagnosed as full WAS (score 3), he had eczema, thrombocytopenia, episodes of bloody diarrhea, and airways infections. Patient W13 (disease score 2-3) is the older brother of patient W12. He presented no clinical symptoms until the age of 5, when he presented with mucocutaneous haemorrhages. He developed mild eczema and, at the age of 12 years, reactive lymphadenopathy. Patient W14 (disease score 5) had thrombocytopenia, mild eczema, autoimmune colitis and vasculitis. He was under prednisone treatment.

Blood samples from WAS patients and healthy donors were obtained according to standard ethical procedure.

3.1.2 T cell lines

Untransformed, polyclonal T cell lines were generated from PBMC of WAS patients and healthy donors as previously described (Dupre et al., 2002). Briefly, PBMC were purified from peripheral blood on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) gradient and seeded at a concentration of 5×10^5 cells/ml. Cells were stimulated in the presence of a feeder mixture composed of 1×10^6 /ml allogenic PBMC (irradiated with 6000 rad by an X-ray source) and 1×10^5 /ml EBV-transformed B cell line JY (irradiated with 10000 rad by an X-ray source), 1 μ g/ml PHA and 100 IU/ml rhIL-2 (Chiron Corp., Emeryville, USA). Cells were cultured in IMDM (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% YSSEL medium (Dyaclone, Besançon, France), 5% FBS (Cambrex Bio Science) and penicillin/streptomycin (Bristol-Myers Squibb, Sermoneta, Italy). T cells were re-stimulated as described every two weeks. CD4⁺ and CD8⁺ T cells subsets were purified by positive selection from established T cell lines, using anti-CD4 or anti-CD8 microbeads, respectively, (Miltenyi Biotech, Bergische Gladbach, Germany) according to the manufacturer's instructions (purity was > 95%).

To generate JY-specific T cell lines, which were used in the cytotoxic assay, PBMC from WAS1 and two normal donors were purified from peripheral blood on Lymphoprep (Nycomed Pharma) gradient and seeded at a concentration of 5×10^5 cells/ml. Cells were stimulated in the presence of 1×10^5 /ml EBV-transformed B cell line JY (irradiated with 10000 rad by an X-ray source) with 40 IU/ml IL-2. CD8⁺ T cells subsets were purified by positive selection from established T cell lines, using anti-CD8 microbeads (Miltenyi Biotech) according to the manufacturer's instructions. Alternatively, allo-specific T cell lines were generated by stimulating PBMC from patient W1 and from healthy donors with a mixture of irradiated JY cells and PBMC obtained from a single allogenic donor. The culture conditions were the same as described here before.

EBV-transformed B cell line JY and chronic myelogenous leukaemia cell line K562 were maintained in IMDM supplemented with 10 % FBS and penicillin/streptomycin.

3.2 Beads preparation

Coating of anti-CD3 mAb and anti-CD28 mAb on the surface of polystyrene latex beads (Interfacial Dynamic Corp., Portland, USA) was performed by incubating the beads at a concentration of 12×10^6 beads/ml in PBS in the presence of the indicated concentration of anti-CD3 mAb alone, or anti-CD3 plus anti-CD28 mAb. Incubation was performed under constant gentle agitation, at 4°C, overnight. Then, 2% FBS was added to the beads suspension and two wash steps were performed. Non-specific binding sites on the beads were blocked by a 1-hr incubation in complete medium. Coated beads were used immediately or stored at 4°C in complete medium for a maximum of 5 days.

3.3 T cell activation

When indicated, resting T cell lines (9-14 days after feeder) were starved overnight without IL-2. Latex beads were coated with the indicated amount of anti-CD3 and anti-CD28 mAbs (BD Biosciences Pharmingen), as previously described. The beads were eventually re-suspended in the required medium and added to the cells, at ratio of 2 bead per cell. Alternatively, T cells were stimulated with plastic-bound anti-CD3 plus soluble anti-CD28 mAbs. In this case, plates were coated overnight with anti-CD3 mAbs in 0.1M Tris pH 9.5, then washed in PBS. T cells were plated in the presence of 10 µg/ml soluble anti-CD28 mAbs.

3.4 T cell proliferation

Resting WAS and control T cell lines were seeded at 1×10^5 cells/well in a 96-well plate round-bottom with 2×10^5 beads coated with the indicated amount of anti-CD3 and anti-CD28 mAbs, in a final volume of 200 µl. T cell proliferation was analyzed after 72 hr of stimulation, by a 16- hours pulse with 1 µCi/well ^3H -thymidine (Amersham Pharmacia Biotech) followed by harvesting using the 96-well plate harvester (Tomtec, Hamden, USA) and counting by liquid scintillation. All experiments were performed in duplicate or triplicate wells.

3.5 TCR down-regulation

Resting T cells were stimulated with plate-bound anti-CD3 mAb (OKT3) for 4 hs at 37°C, 5% CO₂. Then cells were harvested and TCR surface expression was evaluated by staining with anti-TCRα/β mAb (BD Biosciences). The staining was performed in PBS 0.3% BSA. Analysis of TCR expression was performed by FACS. In

order to minimize TCR internalization and recycling after the 4-hs stimulation, all the procedure was performed on ice.

3.6 Analysis of cytokine production

3.6.1 ELISA

The secretion of cytokines was measured in the supernatants of resting and activated WAS and control T cell lines, which were plated at the initial density of 1×10^5 cells/well. Supernatants were collected at different time points (18 hr for IL-2 and 48 hr for the other cytokines) and the presence of cytokines was detected by standard capture ELISA, which was performed according to the manufacturer's instructions (BD Biosciences Pharmingen). Concentration of cytokines was calculated by parallel measurement of a standard curve, generated using recombinant human cytokines (R&D System).

3.6.2 Cytometric bead array (CBA)

Alternatively, cytokine levels in the supernatants of stimulated cells were assessed using the cytometric beads array (CBA: Beckton Dickinson), according to the manufacturer's instructions. 50 μ l of supernatants were used in this assay. Analysis was performed by FACScalibur.

3.6.3 Intracellular cytokine staining

Resting T cells (1×10^6 /ml) were stimulated either with beads coated with anti-CD3 mAbs plus anti-CD28 mAbs or with 10 ng/ml TPA plus 500 ng/ml ionomycin in complete medium, at 37 °C, in a 5% CO₂ atmosphere. Upon initiation of the experiment, plates were centrifuged for 2 min at 800 rpm. After 3 hr, 10 μ g/ml Brefeldin A (Calbiochem) was added to the wells. After additional 3 hr, T cells were collected and fixed in 2% formaldehyde. After fixation, T cells were permeabilized by 10 min incubation in blocking buffer (PBS 0.3% BSA, 0.1% NaN₃) supplemented with

0.5% Saponin (Sigma Aldrich). Permeabilized T cells were incubated in blocking buffer with 0.5% Saponin in the presence of PE-labelled anti-hIL-4, PE-labelled anti-hIL-2 and FITC-labelled anti-hIFN- γ (BD Biosciences Pharmingen). After washing, cells were analyzed by FACS and data were analyzed by CellQuest software.

3.7 Western Blotting

3.7.1 WASP expression

Cell extracts were prepared from 1×10^6 resting T cells. Briefly, T cells were washed in PBS and resuspended in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40), supplemented with protease inhibitor cocktail (Sigma Aldrich, St-Louis, USA). After 30 min on ice, lysates were cleared by centrifugation and an aliquot of supernatants was used for determining protein concentration with the BCA protein assay (Pierce, Rockford, USA). Laemmli buffer was added to supernatants and, after boiling, equal amounts of proteins were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane and WASP was detected by the anti-WASP polyclonal Ab H-250 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by secondary HRP-conjugated anti-mouse Abs (Dako Cytomation). Control of protein loading was performed by hybridizing the same membranes with an anti-G3PDH mAb (Chemicon, Temecula, CA, USA). Detection was performed using ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, UK).

3.7.2 Analysis of ERK1/2 phosphorylation

CD4⁺ and CD8⁺ T cell lines from WAS patients and from healthy donors were starved for 12 hours in IMDM containing low fetal bovine serum (1%), then stimulated with beads coated with 1 μ g/ml anti-CD3 plus 10 μ g/ml anti-CD28 for the indicated times. Reactions were stopped by wash in cold PBS and immediate lysis in whole-cell lysis buffer. After 30 min on ice, lysates were cleared by centrifugation and an aliquot

of supernatants was used to determine protein concentration with the Bradford protein assay (Biorad). Laemmli buffer was added to supernatants and, after boiling, samples with equal amount of proteins were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane and phosphorylated ERKs were detected by anti-phosphoERK1/2 mAb (Cell Signalling Technologies, Danver, MA). To detect levels of total ERK1/2, membranes were stripped by a 15-min incubation, at room temperature, in Restore Buffer (Pierce Biotechnology, Rockford, USA) and re-probed with anti-ERK1/2 pAb (Cell Signalling Technologies). Incubation with primary Abs was followed by incubation with goat anti-mouse or anti-rabbit HRP Ab, according to the specificity of the primary Abs (Dako Cytomation). Detection was performed by ECL detection system (Amersham Pharmacia Biotech).

3.7.3 Analysis of JNK1/2 and of I κ B α phosphorylation

CD4⁺ and CD8⁺ T cells from WAS patients and healthy donors were starved for 12 hours in low serum (1%), then stimulated with beads coated with 1 μ g/ml anti-CD3 plus 10 μ g/ml anti-CD28 for the indicated times. Cytosolic and nuclear fractions were extracted using NE-PER extraction system (Pierce) in the presence of inhibitors of proteases and phosphatases (Sigma) according to the manufacturers' instructions. For Western Blotting, 20 μ g of cytosolic extracts were boiled in Laemmli buffer and loaded on SDS-PAGE. Upon blotting onto nitrocellulose membrane, levels of phosphorylated JNK2/2 were detected by anti-phosphoJNK1/2 pAb (Cell Signalling Technologies). To detect levels of total JNK1/2, membranes were stripped as indicated here before, and re-probed with anti-JNK1/2 (Cell Signalling Technologies). Phosphorylation levels of I κ B α were detected by anti-phosphoI κ B α (Cell Signalling Technologies). Control of protein loading was performed by hybridizing the same membranes with anti-G3PDH Ab.

3.7.4 NFAT and Fos nuclear levels

To detect nuclear levels of NFAT-1, NFAT-2 and Fos, 5×10^6 CD4⁺ and CD8⁺ T cells from WAS patients and healthy donors were starved for 12 hours in low serum (1%), then stimulated with beads coated with 1 μ g/ml anti-CD3 plus 10 μ g/ml anti-CD28 for the indicated times. Cytosolic and nuclear fractions were extracted using NEPER extraction system (Pierce) in the presence of inhibitors of proteases and phosphatases (Sigma) according to the manufacturers' instructions. For Western Blotting, 12 μ g of proteins were boiled in Laemmli buffer and loaded on SDS-PAGE. Upon blotting onto nitrocellulose membrane, NFAT-1 was detected by anti-NFAT-1 T2B1 Ab (a kind gift of A. Rao and S. Feske) and NFAT-2 and Fos were detected by anti-NFAT-2 mAb clone 7A6 and anti-Fos k-25 pAb, which recognizes c-Fos, Fos-B, Fra-1/2 (Santa Cruz Biotechnology). Protein normalization and quality of cell fractionation were assessed by re-probing the same nitrocellulose membranes with an anti-Sp1 pAb (Santa Cruz Biotechnology) for nuclear extracts, and with an anti-G3PDH mAb (Chemicon) for cytosolic extracts. Densitometric analysis was performed by ImageQuant software. Values corresponding to unstimulated cells were subtracted.

3.8 Transduction of T cell lines and PBMC

3.8.1 Production of retroviral vectors

The retroviral vector, used in this study (LDSW), was generated by I.J. Molina starting from the LDNSN vector (Mavilio et al., 1994). Briefly, the WASP cDNA sequence was cloned by PCR using the PCRTM3 plasmid (Invitrogen) containing WASP cDNA (kindly provided by O. Parolini) as a template. To generate the LDSW vector (LTR-dNGFR-SV40-WASP), the LDNSW vector was digested with HindIII plus NaeI restriction enzymes, to excise the Neomycin resistance (NeoR) gene. The digested vector was purified from agarose gel and its termini were filled using the Klenow

fragment of *E.Coli* DNA polymerase I to generate blunt ends. WASP cDNA was then ligated into the vector downstream of the SV40 promoter. A stable amphotropic Am12-based producer cell line (Markowitz et al., 1988) was generated as follows: LDSW plasmid was transfected into GP+E86 ecotropic packaging cell line by Ca_2PO_4 precipitation. Supernatant from transfected GP+E86 cells was used to infect Am12 cell lines, in the presence of 8 $\mu\text{g/ml}$ polybrene. Transduced Am12 cells (AM12-LW) were analyzed by FACS for dNGFR expression using an anti-dNGFR biotin-conjugated mAb (ATCC, Rockville, MD), followed by streptavidin-PE. Cells expressing dNGFR were immunoselected after staining with anti-dNGFR mAb, using rat anti-mouse conjugated magnetic beads (Dynabeads M-450; Dynal, Oslo, Norway). To collect viral supernatants, AM12-LW were trypsinized and plated in fresh medium at a concentration of $5 \times 10^4/\text{cm}^2$. After 48 hs, medium was replaced and the volume was scaled-down to 6 ml in a T75 flask. Supernatants was collected in three rounds (every 12 hours), starting from 24 hs after the last change of medium. Supernatants were filtered with a 0.45 μm filter and immediately frozen at -80°C . As control, the SFCMM3 vector (clone#16) (LTR-tk/SV40-dNGFR) was used (Verzeletti et al., 1998).

3.8.2 Transduction of T cell lines with retroviral vectors

T cells from WAS patients or healthy donors were stimulated for 48 hs with 1 $\mu\text{g/ml}$ plate-bound anti-CD3 mAb, plus 1 $\mu\text{g/ml}$ soluble anti-CD28 mAb, in the presence of 100 IU/ml IL-2 (Chiron Corporation) and 10 ng/ml IL-7 (R&D Systems). After pre-stimulation, cells were collected and plated (10^6 cells/well in a 24-well plate) on retronectin-coated plates. Two rounds of transduction were performed, the first of 24 hs and the second of 6 hs, as previously described (Pollok et al., 1998). Three days after transduction, DNGFR expression was analyzed by FACScan. T cells expressing dNGFR were immunoselected as described in the previous section.

3.8.3 Production of lentiviral vectors

To construct third generation self-inactivating (SIN) lentiviral vectors, WASP cDNA was extracted from the LXSDN plasmid by digestion with BbsI and BamHI. WASP fragment was blunted and inserted into the pBluescriptII (KS) plasmid digested with EcoRV. The PGK-WASP construct was generated by inserting the WASP cDNA fragment (obtained by digestion with the enzymes BamHI and SalI) into the pRRLsin.cPPT.PGK.E-GFP.Wpre plasmid (PGK-GFP) (Follenzi et al., 2000), in place of the E-GFP cDNA fragment. The construct containing WASP autologous promoter (wasp-WASP) was obtained by removal of the PGK promoter, followed by insertion of the 5' promoter region of the *WAS* gene (-1580/+33), kindly provided by Dr. Morrone (Petrella et al., 1998). Production of lentiviral vectors was performed as previously described (Follenzi et al., 2000). Briefly, WASP-encoding transfer plasmids were transfected together with the three packaging plasmids (encoding gag-pol, VSV-G env and rev, respectively) into 293T cells by CaCl₂ precipitation. After 14 hs, culture medium was replaced by fresh medium. A single harvest of viral supernatants was performed after 24 hs, and virus particles were concentrated by ultracentrifugation. Virus concentration was evaluated by measurement of p24 by ELISA.

3.8.4 Transduction of T cell lines and of PBMC with lentiviral vectors

T cells from WAS patients or healthy donors were stimulated as described in section 3.8.2. After 48 hs of pre-stimulation, cells were collected and plated at a density of 10⁶ cells/ml. Alternatively, PBMC were stimulated according to a method previously published (Cavaliere et al., 2003), with the following modifications: PBMC were stimulated for 96 hs with 60 IU/ml IL-2 plus 5 ng/ml IL-7, before transduction. Transduction was performed in the presence of 8 µg/ml polybrene (Sigma) at the indicated concentration of TU/ml for the PGK-GFP vector or at a concentration of 100

ng p24/10⁵ cells in a volume of 0.5 ml for PGK-WASP and wasp-WASP vectors. A single round of 24 hs of transduction was performed.

3.9 T cell adhesion

To analyze adhesion to anti-CD3 mAb or fibronectin, resting T cell lines from patients W1 and W2 and from three healthy donors were loaded with 5 mM Calcein-AM (Molecular Probes, Inc., Eugene, OR). Briefly, cells were resuspended in PBS with Calcein-AM and incubated at 37°C, for 30 min. Then, an equal volume of PBS, 10% FBS was added to the cells. After 10 min, cells were washed and resuspended in adhesion medium (RPMI without phenol red, 0.5% human serum albumin, glutamine). Anti-CD3 mAb (escalating doses) or fibronectin (5 µg/ml) (Sigma) were coated in 96-well plates by a 12-hs incubation in PBS, followed by washing in PBS and by a 2-hs blocking in PBS, 1% BSA. Cells were left to adhere for 30 minutes, at 37°C, 5% CO₂, then the fluorescence was analyzed by Typhoon (“pre”). Subsequently, non-attached cells were removed by a 5 min centrifugation, up side down, at 150g. Then, 100 µl of adhesion medium were added to the cells and Calcein fluorescence was analyzed by Typhoon (“post”). All the experiments were performed in triplicates. Fraction of adherent cells was calculated as follows: $1 - ((\text{pre value} - \text{post value}) / \text{pre value})$.

3.10 GM1 and GM3 expression

To evaluate the levels of surface GM1 and GM3, resting T cell lines from WAS patients and healthy donors were stimulated as indicated, with beads coated with anti-CD3 mAb alone, or plus anti-CD28 mAb, or, alternatively, with plate-bound anti-CD3 mAb alone, or plus soluble anti-CD28 mAb. After 72 hs of stimulation, cells were collected and GM1 and GM3 surface expression was evaluated after staining with Cholera Toxin B subunit FITC (Sigma) or with anti-GM3 mAb, clone GM36

(Seikagaku Corporation, Tokyo, Japan), followed by goat anti-mouse IgM PE conjugated, respectively. Cells were then analyzed by FACScan.

In order to evaluate the levels of total cellular GM1, after stimulation, cells were fixed overnight in 2% formaldehyde in PBS, at 4°C. After washing in PBS, 0.3% BSA, cells were permeabilized with PBS BSA plus 0.5% Saponin, for 10 min at room temperatures. Staining was performed with Cholera Toxin B Subunit FITC, in the presence of 0.5% Saponin, for 30 minutes at room temperature. Analysis was performed using a FACScan.

3.11 Analysis of Ca²⁺ flux

Ca²⁺ flux upon TCR triggering was analyzed in resting T cell lines from patients W1 and W2 and from two healthy donors. Cells were resuspended in KRH solution (125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 25 mM HEPES pH 7.4, 2 mM CaCl₂, 6 mM Glucose) and a 1:1 mixture of 4 µM Fluo3-AM (Molecular Probes) and Pluronic Acid was added to the cells. Loading of Fluo3 was achieved by a 30-min incubation at room temperature, under gentle agitation. Cells were then washed with KRH in the presence of the anion transporter inhibitor Sulfynpirazone (Sigma), at 200 µM. Stimulation was performed in KRH. Anti-CD3 mAb (1 µg/ml) was added to the cells on ice. After 15 min, cells were analyzed by FACS. Fluorescence was recorded for 20 sec ca., then cell-bound anti-CD3 mAb was crosslinked by the addition of 5 µg/ml of goat anti-mouse Ab. Fluorescence was recorded for 6 minutes, then 1 mM ionomycin was added to the cells. Analysis was performed using CellQuest Software, calculating the mean fluorescence intensity (MFI) of the cells population, in adjacent gates of 10 sec ca. each.

3.12 RNase protection assay (RPA)

Resting T cells were stimulated by 1 µg/ml of immobilized anti-CD3 mAbs plus 10 µg/ml soluble anti-CD28 mAbs or by 10 ng/ml TPA plus 500 ng/ml ionomycin. After 4 hr of incubation at 37 °C, in a 5% CO₂ atmosphere, cells were collected and RNA was extracted as previously described (Guidotti et al., 1999). Total RNA extracted from either CD4⁺ or CD8⁺ T cells was analyzed for the content of the messages for IFN-γ, IL-2, IL-4 and IL-10 by using the highly sensitive multi-probe RNase protection assay (RPA). Briefly, probe synthesis was driven by T7 bacteriophage RNA polymerase with α-[³²P]UTP as the labelling nucleotide. The subsequent steps of probe purification, RNA-probe hybridization, Rnase treatment, purification of protected RNA duplexes, and resolution of protected probes by denaturing PAGE was performed as described (Hobbs et al., 1993). Probe bands were visualized by autoradiography and quantified by phosphor imaging analysis, using the ImageQuant analysis software (Amersham Biosciences). For the latter, rectangular fields (with local background subtraction) were used for volume quantification. The relative abundance of mRNA species was obtained dividing the value of each field by the relative L32 value of that sample in order to obtain a relative expression value for each individual mRNA species. Values corresponding to unstimulated cells were subtracted.

3.13 T-bet and GATA-3 mRNA expression

To measure T-bet and GATA-3 mRNA levels, CD4⁺ T cell lines from WAS patients and healthy donors were stimulated with immobilized anti-CD3 mAbs plus 10 µg/ml soluble anti-CD28 mAbs for four hours. Total RNA was extracted from 1×10⁶ cells using Eurozol (Euroclone S.p.A., Milan, Italy) and RNA was reverse transcribed by using the High Capacity cDNA Archive Kit (Applied BioSystems, Foster City, CA,

USA) according to the manufacturer's instructions. T-bet and GATA-3 mRNA species were quantified using the Assay on Demand kit (Applied Biosystems) and Taqman Master Mix (Applied Biosystems). Each sample was analysed in duplicate and the relative expression of T-bet and GATA-3 was defined by calculating the ΔC_t , with respect to the housekeeping gene HPRT, run in the same tube.

3.14 Cytotoxic assay

Cytotoxic activity of CD8⁺ T cells was assessed by a standard ⁵¹Chromium (⁵¹Cr) release assay as previously described (Fleischhauer et al., 1996). Briefly, after a 2-min slow-speed centrifugation, 500 ⁵¹Cr-labeled target cells (JY cell line) and effector CD8⁺ T cells, at the indicated effector:target ratio, were incubated for 4 h at 37 °C, in 5% CO₂. Exhaustion of NK-like activity was obtained by 45-min incubation of effector cells with not-radiolabeled (cold) K562 cells, before adding radiolabeled JY cells. Alternatively, T cell blasts obtained by a 5 days culture in the presence of 100 IU/ml IL-2 and 1 µg/ml PHA were used as target cells. Radioactivity released in the supernatants by lysed target cells was measured with a γ-counter. Percentage of specific lysis was calculated according to the following formula: $100 \times (\text{cpm experimental release} - \text{cpm spontaneous release}) / (\text{cpm maximum release} - \text{cpm spontaneous release})$.

3.15 Differentiation of Th1 cells

Naïve (CD45RO⁻) CD4⁺ T cells were purified from PBMC of W1 and two age-matched healthy donors by negative selection, using the CD4⁺ T cells isolation kit according to the manufacturer's instructions (Miltenyi Biotech). Naïve CD4⁺ T cells were obtained by depleting CD45RO⁺ cells with anti-CD45RO Myltenyi beads (Miltenyi Biotech), according to the manufacturer's instructions (purity ranged from 85 to 95%). Naïve CD4⁺ T cells were activated by co-culture with murine L cells, stably

transfected with hCD32 (FC γ RII), hCD58 (LFA-3), and hCD80 (de Waal Malefyt et al., 1993). L cells were trypsinized and X-ray irradiated (7000 rad). Following washing, cells were seeded at 4×10^5 cells/well in a 24-well plate in IMDM supplemented with 10% FBS, penicillin/streptomycin and 100 ng/ml anti-CD3 mAbs (Orthoclone OKT3, Janssen-Cilag, Milan, Italy). After adhesion of L cells, 4×10^5 naïve CD4⁺ T cells were plated in either non-polarizing (Th0) conditions, or in the presence 10 ng/ml rhIL-12 (R&D systems, Minneapolis, USA), and 200 ng/ml anti-hIL-4 mAbs (BD Biosciences Pharmingen, San Diego, USA) for Th1 polarization. At day three, 40 IU/ml IL-2 was added to all culture conditions. After one week, cells were restimulated with either 10 μ g/ml plate-bound anti-CD3 mAbs plus 1 μ g/ml soluble anti-CD28 mAbs, or with 10 ng/ml TPA (Calbiochem Biochemicals, La Jolla, USA) plus 500 ng/ml ionomycin (Calbiochem Biochemicals) and analyzed for the profile of cytokine production.

3.16 Inhibition of glycosphingolipids synthesis

To inhibit glycosphingolipids biosynthesis, T cell lines established from healthy donors were stimulated with either beads coated with anti-CD3 plus or minus anti-CD28 mAbs, or with immobilized anti-CD3 plus or minus soluble anti-CD28 mAbs, in the presence of the ceramide analogue, L,D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, Sigma Aldrich), used at a concentration of 10 μ M. To test the effect of PDMP, after 72 hours of stimulation, T cell lines harvested and the level of the glycosphingolipid/ganglioside GM1 on the cell surface was assayed by staining with CholeraToxin B subunit-FITC. In some cases the level of another ganglioside, GM3, was measured by surface staining with anti-GM3 mAb as previously described. Cells were then analyzed by FACScan. To test the effect of PDMP on T cell proliferation and IL-2 secretion, T cell lines were loaded with CFSE. Briefly, cells were resuspended in PBS at a concentration of 10^7 /ml in the presence of 2.5 μ M CFSE

(Molecular Probes) and kept under gentle agitation for 8 min in the dark. Extracellular fluorescence was quenched by the addition of PBS containing 5% FBS. CFSE-loaded cells were washed twice and resuspended at a concentration of 10^6 /ml in complete medium. Stimulation was performed with anti-CD3 plus or minus anti-CD28 mAbs, in the presence, or absence, of 10 μ M PDMP. IL-2 level was measured in the supernatants of stimulated cells after 18 hours, by capture ELISA, Cell proliferation was assed by analyzing CFSE dilution after 72 hours of stimulation. Dead cells were excluded from the analysis by a short pre-incubation of Propidium Iodide.

3.17 Isolation of detergent-resistant membranes (DRM) by flotation in a sucrose gradient

In order to analyze the partition of WASP into DRM (non-soluble) or non-DRM (soluble) fractions, resting T cell lines from healthy donors were stimulated for the indicated times with 1 μ g/ml anti-CD3 plus 10 μ g/ml anti-CD28 mAbs, followed by crosslinking with 30 μ g/ml goat anti-mouse Abs (Dako Cytomation) at 37 °C. Stimulation was stopped by washing in cold PBS and lysis in Buffer A (2 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% non-ionic detergent (Brij58), followed by Dounce homogenization and a 45-min incubation on ice. The cell lysate was mixed at a 1:1 ratio with 85% Sucrose in Buffer A and a discontinuous gradient (30-5%) of Sucrose was stratified over the cell lysate, followed by a 16-hours ultracentrifugation at 4 °C in a swing-out SW41 rotor (Beckman Instruments, Inc.). After centrifugation, low density floating fractions (DRM) and high density fractions (non-DRM) were collected. Proteins were precipitated from the entire fractions by addition of trichloroacetic acid and the protein pellet was washed with acetone 73%. After precipitation, protein pellets were directly re-suspended in Laemmli buffer and pH was adjusted by exposure to NH₃ vapours. Proteins were then resolved by SDS-PAGE. Proteins were transferred to

nitrocellulose and WASP, LAT and CD3- ζ were detected by anti-WASP pAb (Upstate Biotechnology), anti-LAT Ab (Santa Cruz Biotechnology) and anti-CD3- ζ Ab (Santa Cruz Biotechnology), respectively. After incubation with the secondary- HRP-conjugates Abs, proteins were detected by chemiluminescence using either SuperSignal West Dura (Pierce) or ECL (Amersham).

3.18 Isolation and characterization of nTreg cells from WAS patients

3.18.1 CD25 and FoxP3 expression

Human PBMCS were purified on Ficoll gradient (Nycomed Pharma A/S, Oslo, Norway) from blood samples of WAS patients and age-matched healthy donors. The following antibodies were used for immunophenotyping of nTregs: anti-CD4 (SK3), anti-CD25 (2A3), all from BD Pharmingen. Foxp3 expression was evaluated by intracytoplasmic staining using the hFoxp3 (PHC101) Staining Set from eBioscience, accordingly to manufacturer's instructions. Analysis of the CD4, CD25 and FoxP3 expression was performed using a FACScalibur flow cytometer.

3.18.2 *In vitro* suppression assay

CD4⁺CD25⁺ nTregs and CD4⁺CD25⁻ effector T cells were isolated from PBMCS by FACS sorting. Cells from healthy donors were used as control: CD4⁺ T cells were purified either by FACS sorting or, alternatively, by negative selection with the CD4⁺ T cell isolation kit (Miltenyi Biotec). Subsequently, cells were separated into CD25⁺ and CD25⁻ fractions by positive selection (Miltenyi Biotec). In both cases, purity of nTreg cells ranged from 80 to 95% and purity of CD4⁺CD25⁻ effector T cells was $\geq 95\%$. Suppression assays with purified human nTregs were performed as follows: 5×10^4 CD4⁺CD25⁻ effector T cells were plated in 96-well round bottom plates together with nTregs and stimulated by CD3-depleted APCs (irradiated at 6000 rad) and soluble anti-CD3 mAbs (OKT3, 1 $\mu\text{g/ml}$) in a final volume of 200 μl . Alternatively, when very low

numbers of nTregs were recovered, 1×10^4 effector T cells were cocultured with nTregs and stimulated with beads coated with anti-CD3 and anti-CD28 mAbs (DynaBeads), at a responder:bead ratio of 1:0.6. Suppressive activity of nTregs was assessed by co-culture of effector cells with nTregs at a ratio of 2:1 or 4:1. After 72 hours of culture, cell proliferation was evaluated by liquid scintillation counting, and IFN- γ secretion was evaluated on supernatants using the human Th1/Th2 cytokine cytometric bead array (CBA) system (BD Biosciences). Samples were analysed on a BD FACSCalibur flow cytometer, according to manufacturer's instructions. Values of IFN- γ in the supernatants were normalized to a number of 2×10^5 cells per 200 μ l.

4 RESULTS

4.1 WASP lowers the threshold for TCR/CD28-driven T cell activation

4.1.1 Bulk T cell lines established from WAS patients PBMC show defective proliferation in response to anti-CD3 mAb

WASP-deficient T cells from WAS patients and WASP knock-out mice showed defective proliferative response to anti-CD3 mAb (Molina et al., 1993; Snapper et al., 1998; Zhang et al., 1999). To further characterize the extent of this proliferative defect and to clarify the role of WASP in TCR/CD28 responsiveness, untransformed T cell lines were established from three WAS patients, with different clinical phenotype (Table 1). Western Blot analysis showed that in resting T cells from WAS patients WASP expression was undetectable (Figure 1A). However, upon stimulation with anti-CD3 mAb low levels of full-length WASP were detected in T cells established from patient W3, (Figure 1B), who has a missense mutation which can be compatible with the expression of residual, point-mutated, WASP protein. WAS T cells proliferated poorly to immobilized anti-CD3 mAb (Figure 2A). In particular, stimulation with low dose of anti-CD3 mAb (0.1 μ g/ml) was able to induce maximal proliferation of control cells, while it failed to elicit proliferation in WAS T cells. However, WAS T cells could proliferate to relatively high levels when high concentration of anti-CD3 mAbs was used (Figure 2A). Despite defective proliferation, WAS T cells retained normal ability to down-regulate surface expression of the TCR upon stimulation with immobilized anti-CD3 mAb (Figure 2B).

Since WASP is required for actin polymerization and it is recruited to the site of contact between T cell and APC (Cannon et al., 2001), it is possible that WASP plays a role in the establishment of cell-cell conjugates. Therefore, we tested the proliferative response using beads coated with anti-CD3 mAb or antiCD3 plus anti-CD28 mAb.

Such a stimulation was used to mimic T cell-APC contact and was shown to induce both polarization of lipid rafts and T cell proliferation (Krause et al., 2000; Viola et al., 1999). WAS and control T cell lines were stimulated with beads coated with escalating doses of anti-CD3 mAb alone (Figure 2C), or in the presence of 10 μ g/ml anti-CD28 mAb (Figure 2D). Proliferation of WAS T cells was reduced with respect to control cells upon stimulation with anti-CD3 mAb (Figure 2C). The addition of anti-CD28 to anti-CD3 mAbs greatly enhanced the proliferative response of normal T cells (Figure 2D), while proliferation of WAS T cells was significantly lower ($p < 0.05$ at all doses of anti-CD3 tested for patients WAS1 and WAS2, $p < 0.05$ for patients WAS3 at the doses of 0.01 and 0.1 μ g/ml anti-CD3 mAbs). These data indicate that TCR- or TCR/CD28-mediated proliferation of WAS T cells is defective, especially in conditions of suboptimal TCR-mediated stimulation. This defect is not due to impairment in the ability to internalize the triggered TCRs.

Table 1

Patient	Disease (score^a)	Age^b (years)	Mutation
W1	WAS (4)	24	Deletion (+2→+5 tgag) Intron 9, donor splice site
W2	WAS (5)	5	Deletion (484 ag) Exon 4, STOP at codon 167
W3	XLT (2)	1	Missense (t348→c), Exon 3, K 105→P
W4	WAS (5)	23	Deletion (150 t) Exon 6, STP at codon 260
X6	XLT (0.5)	10	Missense (c207→g) Exon 2, P58→R
W8	WAS (3)	2	Insertion (+3 t), Intron 8, donor splice site
W11	WAS (5)	1	Missense (c435→t) Exon 4, A134→V
W12	WAS (3)	11	Missense (t150→c), Exon 1, L 39→P
W13	WAS (2-3)	14	Missense (t150→c), Exon 1, L 39→P
W14	WAS (5)	2	Missense (g397→a), Exon 4, E 121→K

^a Disease score is given according to the classification by Zhu Q. et al., 1997 and Jin Y. et al., 2004

^b Age refers to the time of blood sampling

Figure 1

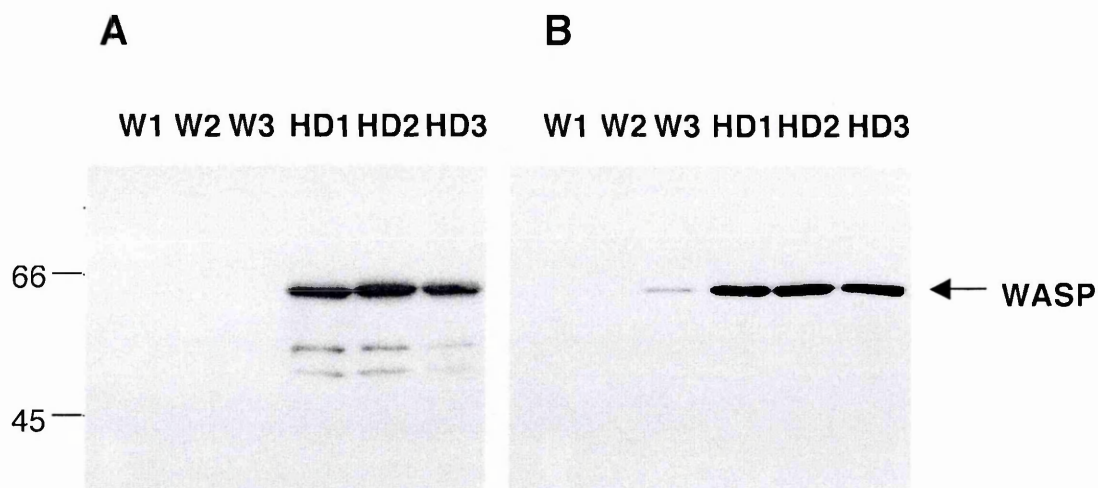


Figure 1. Defective WASP expression in T cell lines from WAS patients. T cell lines from healthy donors (HD) and WAS patients in resting state (A) or after 24 hours of stimulation with 10 µg/ml immobilized anti-CD3 mAb (B) were analysed for WASP expression by Western Blot.

Figure 2

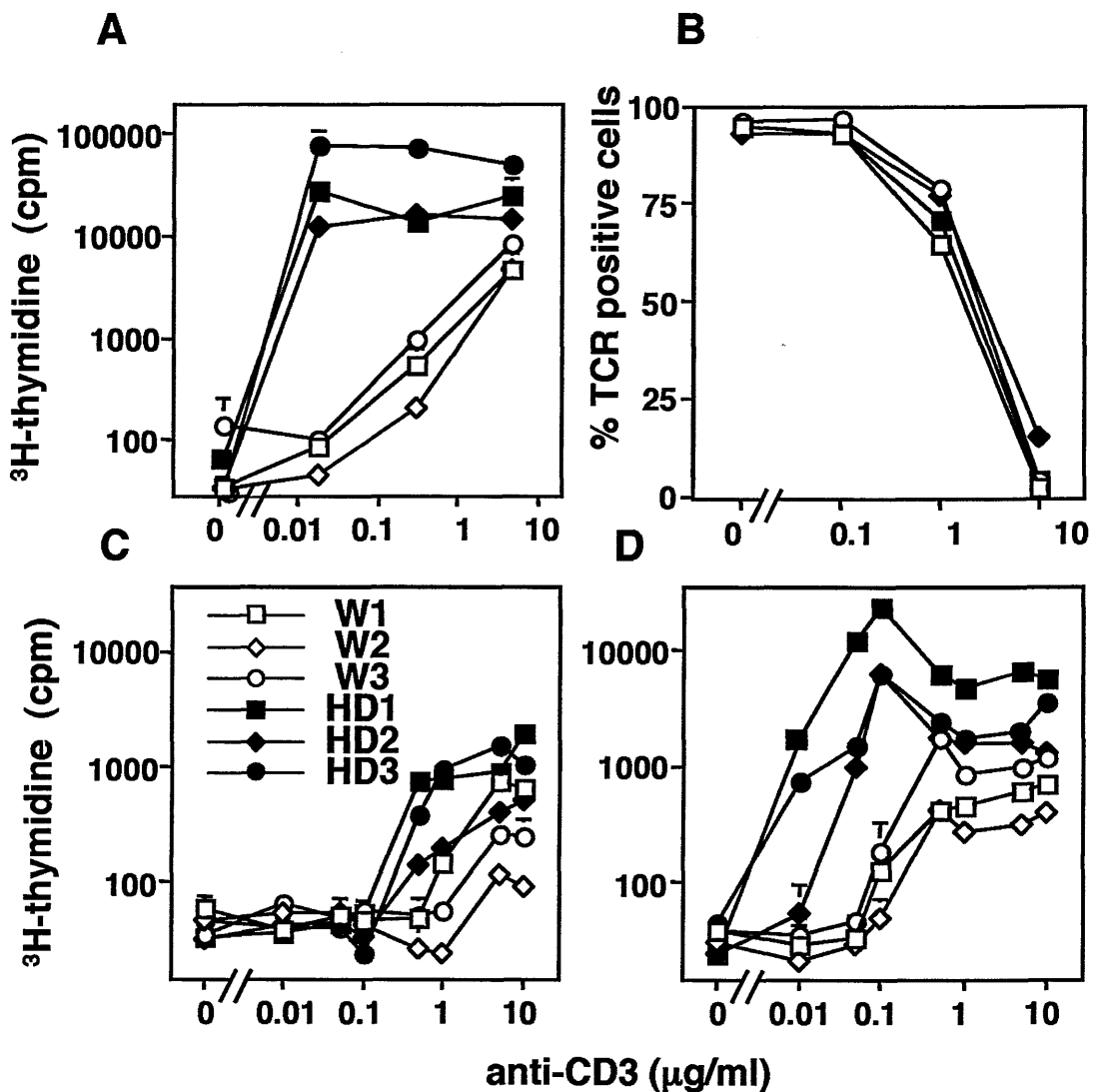


Figure 2. Defective proliferation of WAS T cell lines after TCR and TCR/CD28 triggering. T cell lines from healthy donors (HD: filled symbols) and WAS patients (W: empty symbols) were stimulated with increasing doses of immobilized anti-CD3mAb (A). The rate of TCR downregulation was analyzed in WAS and control cells after 4 hours of stimulation with immobilized anti-CD3 mAb (B). Alternatively, cells were stimulated with bead coated with the indicated doses of anti-CD3 mAb only (C) or anti-CD3 plus anti-CD28 mAb (D). Proliferation was evaluated by ^3H -thymidine incorporation after 72 hours of stimulation. Values represent triplicate cultures. Error bars represent standard deviation. One representative experiments out of five is shown.

4.1.2 CD4⁺ and CD8⁺ T cell lines from WAS patients show defective proliferation in response to TCR/CD28-mediated stimulation

Our T cell cultures contain a mixed population of CD4⁺ and CD8⁺ T cells, with a preferential selection in favour of CD4⁺ T cells in cultures from WAS patients (not shown). These two cell subsets can have intrinsic differences in their response to anti-CD3 mAb stimulation, which could derive from different threshold required for activation, response to co-stimulation or even susceptibility to activation induced cell death (AIDC). In addition, it is possible that in a mixed culture the response of one cell subset is influenced by the other one, by expression of membrane molecules or by release of soluble factors. Therefore, we decided to perform experiments using purified CD4⁺ and CD8⁺ T cell lines, in order to clarify the relative contribution of CD4⁺ and CD8⁺ T cells to the proliferative defect observed in WASP-deficient T cell lines.

CD4⁺ and CD8⁺ T cell lines were established from 4 WAS patients (W1, W2, W8, W12) and 1 XLT patient (X6) (see Table 1) and from healthy controls. Analysis of WASP expression in resting T cells showed that the levels of WASP in CD4⁺ T cells from WAS patients were almost undetectable (Figure 3A and 3B), while a very faint band corresponding to full-length WASP was present in WAS CD8⁺ T cells (Figure 3B). The results are different from what we previously observed in bulk T cells from patients W1 and W2, where WASP was undetectable by Western Blot (Figure 1). It should be noted that a different anti-WASP Ab was used to evaluate WASP expression in bulk T cells. Expression of residual full-length WASP could be expected in CD8⁺ T cells from patient W12, who has a missense mutation, but not in T cells from patient W2, who has a deletion leading to a premature STOP codon. A possible explanation could be cross-reactivity of the Ab with the WASP homologue N-WASP, whose size is similar to the one of WASP. T cells from the XLT patient showed residual WASP expression that was clearly detectable in both CD4⁺ and CD8⁺ T cells (Fig. 3A and 3B).

FACS analysis showed that CD4⁺ and CD8⁺ T cells from the XLT patient were uniformly WASP^{dim} (not shown), indicating that the mutated WASP is expressed at comparable levels in all cells. Upon stimulation with beads coated with a fixed dose of anti-CD28 mAb and escalating doses of anti-CD3 mAb, both CD4⁺ and CD8⁺ T cells from WAS patients showed defective proliferation at all doses of anti-CD3 mAb tested, however, proliferation of WAS T cells was more affected at low anti-CD3 mAb doses (Figure 3C and 3D). Interestingly, T cells from the XLT patient displayed an intermediate level of proliferation (Fig. 3C and 3D). A more severe proliferative defect in CD8⁺ T cells, compared to CD4⁺ T cells, was reproducibly observed in patients X6, W1 and W2 while CD4⁺ and CD8⁺ T cells from patients W8 and W12 showed a comparable defect in proliferation.

These results indicate that both CD4⁺ and CD8⁺ T cells require WASP for TCR/CD28 induced proliferation and suggest that WASP expression levels correlates with the ability of CD4⁺ and CD8⁺ T cells to respond to TCR/CD28-mediated stimulation. In addition, data presented here suggest that WASP plays a crucial role in lowering the threshold for TCR/CD28-driven proliferation.

Figure 3

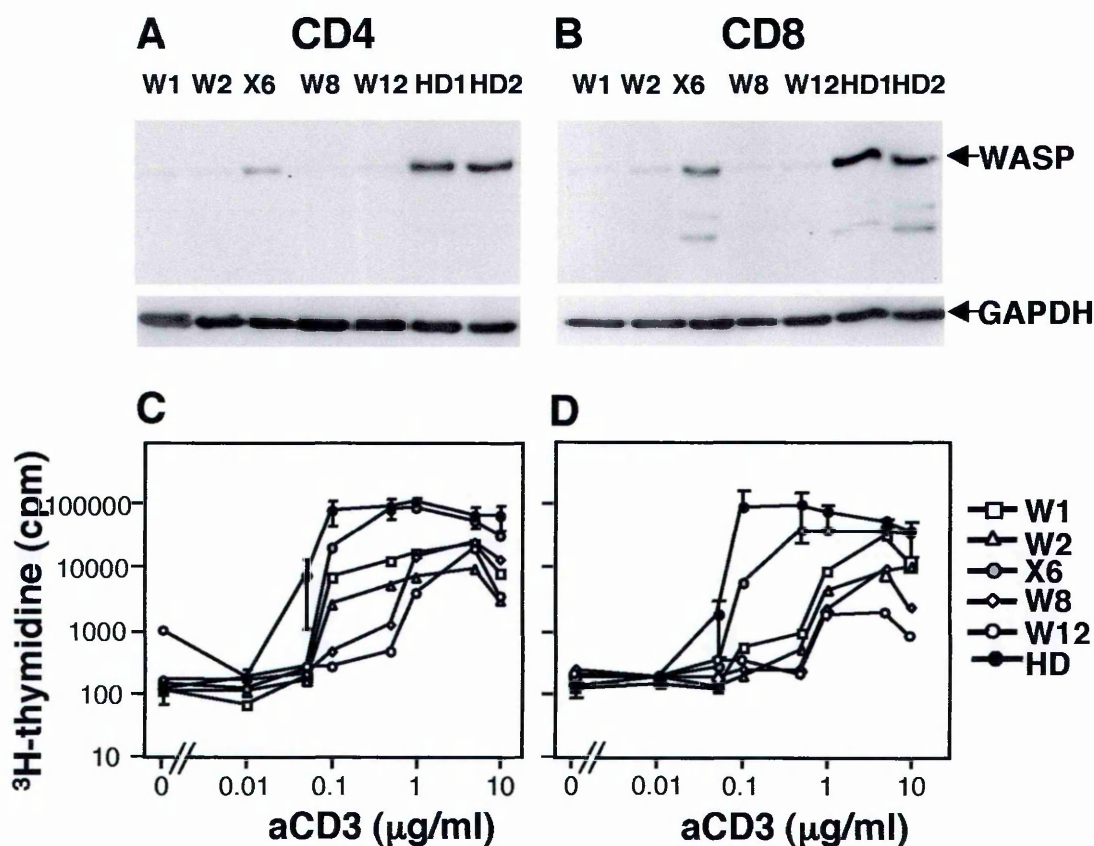


Figure 3. WASP expression and proliferation of CD4⁺ and CD8⁺ T cell lines from WAS/XLT patients and healthy donors. Western Blot analysis of WASP expression in resting CD4⁺ (A) and CD8⁺ (B) T cells from WAS and XLT patients (W1, W2, X6, W8 and W12) and from healthy donors (HD1 and HD2). Proliferation of CD4⁺ (C) and CD8⁺ (D) T cells from healthy donors (n=4) and WAS/XLT patients was measured by ^3H -thymidine incorporation, after 72 hours of stimulation with beads coated with the indicated doses of anti-CD3 plus 10 $\mu\text{g/ml}$ anti-CD28 mAb. Values represent mean of duplicate cultures and are representative of one out of three independent experiments including all patients and out of six experiments involving patients W1 and W2. Error bars in the HD line represent standard deviation of n=4 healthy donors' proliferation values.

4.1.3 LV-mediated WASP gene transfer restores physiological WASP expression and rescues proliferation to anti-CD3 mAb in WAS T cell lines

In order to verify whether WASP deficiency is directly responsible for reduced proliferation to anti-CD3/CD28 mAb of WAS T cells, RV were used to transfer WAS cDNA into T cell lines from WAS patients. LDNSW vector, derived from the LNSN vector (Mavilio et al., 1994), contains WAS cDNA under the control of the SV40 promoter and the reporter gene dNGFR under the control of the viral LTR (Figure 4A). Efficiency of transduction of activated WAS T cells, evaluated by dNGFR expression, was very low (around 3%: not shown). Therefore, in order to enrich for the proportion of transduced cells, immunoselection was performed, achieving more than 85% of dNGFR⁺ cells (not shown). WASP expression was then evaluated in resting WAS T cells transduced with LDNSW vector (Figure 4B), showing that expression of full-length WASP was present, although at lower levels than in control cells. At the time these experiments were performed, it was not possible to evaluate the percentages of WASP⁺ cells, however, the results obtained here indicate that the RV used was not able to induce physiological levels of WASP expression, despite the fact that the vast majority of the cells were transduced.

Analysis of proliferative response to beads coated with anti-CD3 plus anti-CD28 mAb showed that WASP expression significantly enhanced the proliferation of WAS T cells with respect to their untransduced counterparts ($p < 0.01$ t test for the anti-CD3 mAb doses of 0.1 and 1 $\mu\text{g/ml}$), while transduction with control RV (containing only dNGFR cDNA) did not significantly affect the proliferation of W1 cells (Figure 4C). However, the proliferation of WASP-transduced W2 T cells remained below the normal levels at all anti-CD3 mAb doses tested, indicating that in these cells proliferation was only partially corrected. Indeed, WASP-transduced T cells from both W1 and W2 patients failed to proliferate in response to the lowest anti-CD3 mAb dose (0.01 $\mu\text{g/ml}$).

Interestingly, WASP-transduced T cells from patient W3 showed complete correction of the proliferation (Figure 4C). It should be noted, however, that W3 T cells, when activated through the TCR, were able to induce expression of endogenous WASP, although at low levels (Figure 1B).

These results indicate that RV vectors are able to restore WASP expression in T cell lines from WAS patients. However, only low levels of WASP expression were detected in WASP-transduced T cells from WAS patients, which is probably the cause of the only partial rescue of the proliferation.

An alternative approach based on the use of LV was tested to transfer *WAS* gene into WAS T cell lines. LV were chosen in order to improve the efficiency of transduction. Although no data are available regarding fine-tune regulation of WASP expression related to the state of maturation or of cell activation, it is possible that constitutive WASP expression results in a toxic effect to the cells. For these reason, the possibility of driving *WAS* cDNA expression by using *WASP* gene endogenous 5' regulatory elements was evaluated in this study.

Two different third-generation, SIN HIV-derived LV vector were constructed, in which *WAS* cDNA was put under the control either of the ubiquitous PGK promoter (PGK-WASP) or of the endogenous *WASP* promoter (Wasp-WASP) (Figure 5A). As control, a vector containing the green fluorescent protein (GFP) under the control of PGK promoter (p-G) was used (Follenzi et al., 2000) (Figure 5A). TCR/CD28-activated W1 T cell line was transduced with WASP-encoding LV vectors and WASP expression was analyzed by FACS, showing a close-to-normal expression and high percentages of transduction (Figure 5B). WASP expression driven by LV was able to restore normal proliferation in W1 T cell lines stimulated via the TCR, at all doses of anti-CD3 mAb tested (Figure 5C).

Figure 4

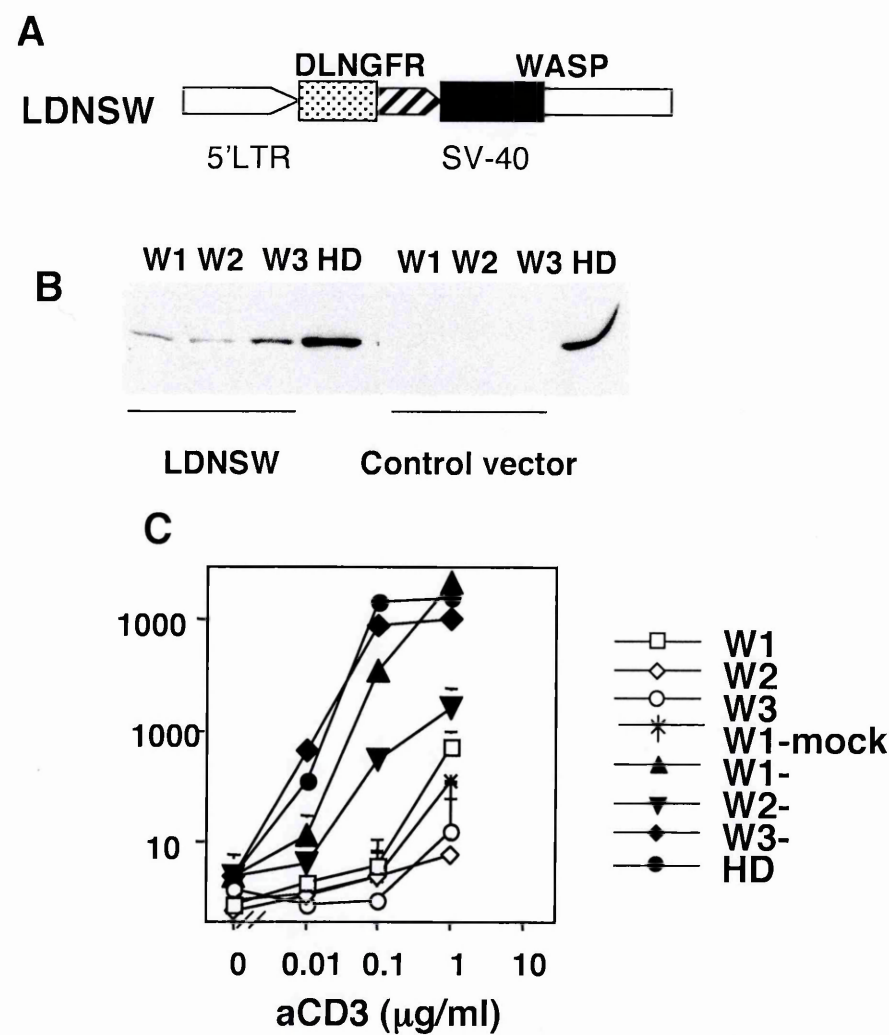


Figure 4. Transduction of T cell lines from WAS patients with MMLV-derived retroviral vectors. MMLV-derived RV (LDNSW) is shown in figure 4A. WASP expression was driven by SV40 promoter, dNGFR was used as reporter gene and put under the control of the viral LTR. Western Blot analysis of WASP expression in whole cell lysate of WAS T cell lines after transduction with LDNSW RV and immunoselection (B). As control, WASP expression level in one T cell line from healthy donor is shown. Proliferation in response to stimulation with beads coated with anti-CD3 plus anti-CD28 mAb was evaluated in RV-transduced T cells from WAS patients (W-tr). As control, proliferation of untransduced WAS T cells (UT: empty symbols) and of W1 T cells transduced with a control vector (mock) was shown (C). Values correspond to triplicate cultures. Error bars represent standard deviation. One representative experiment out of five is shown.

Figure 5

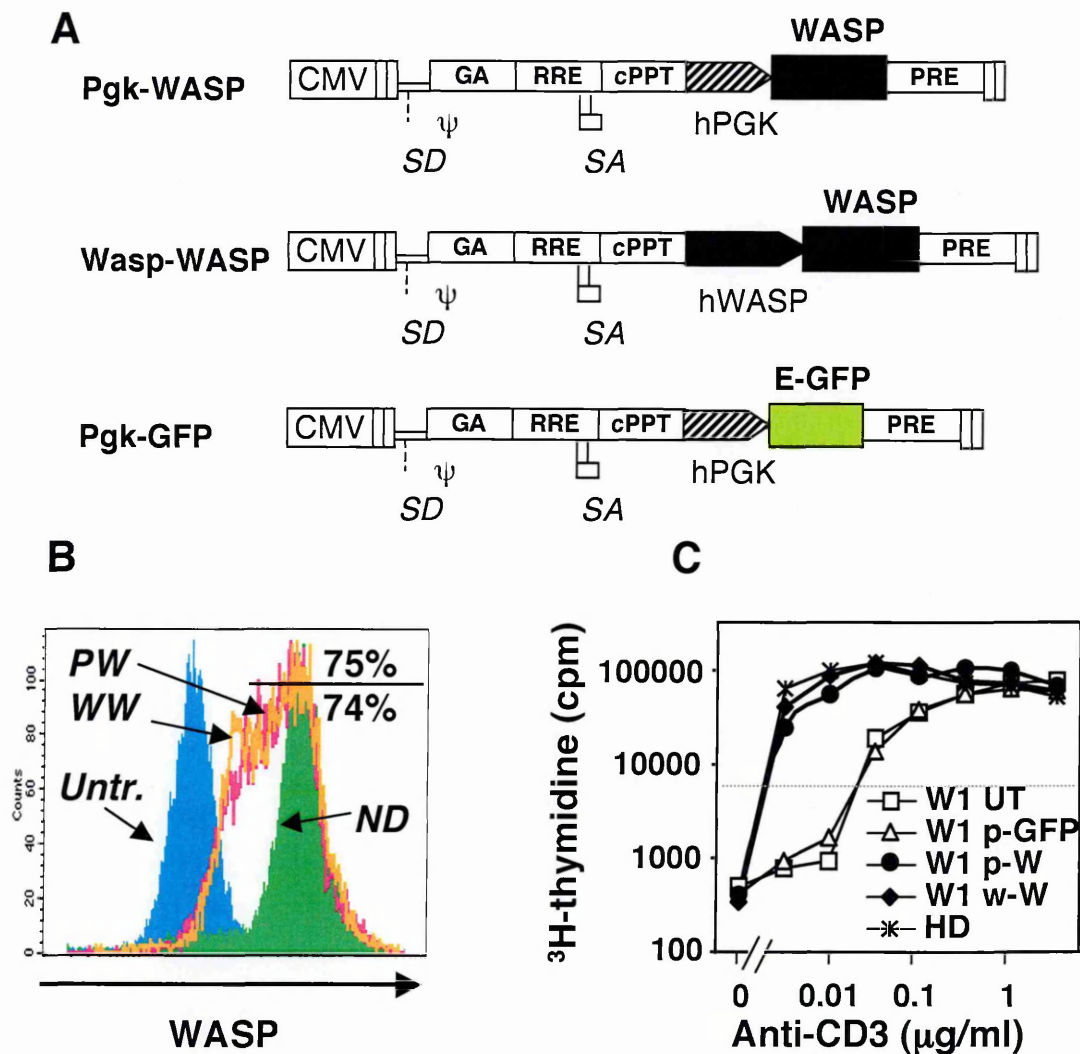


Figure 5. Transduction of T cell lines from WAS patients with HIV-derived lentiviral vectors. Three different types of HIV-derived retroviral vectors are shown in figure 6A. WASP expression was driven by either PGK promoter (Pgk-WASP/p-W) or by the autologus WASP promoter (Wasp-WASP/w-W). As control, a lentiviral vector encoding for GFP was used (pgk-GFP/p-GFP). WASP expression in W1 T cell lines was analyzed by FACS six weeks after transduction with the indicated LV vectors. As control, WASP expression level in untransduced T cell lines from W1 and one healthy control is shown (B). Cell proliferation was measured by ^3H -thymidine incorporation in T cell lines from patient W1 either untransduced, transduced with p-GFP or with the two WASP-encoding LV. As control, the mean proliferation of two healthy donors' T cell lines (HD) is shown (C). One representative experiment out of three is shown. Values correspond to triplicate cultures.

These results indicate that LV allow high efficiency of transduction and close-to-normal restoration of WASP expression in WAS T cells. Importantly, endogenous WAS promoter was as efficient in driving WASP expression and in correcting the proliferation, as the constitutive PGK promoter. Therefore, the use of LV containing autologous WASP promoter seems to be the best approach to transduce mature WAS T cells, which could represent a possible target of gene therapy in some WAS patients. Based on the results obtained in human T cell lines, these LV were also evaluated in their ability to mediate WAS gene transfer into repopulating murine WASP-deficient HSC. Results obtained from experiments performed in WAS murine model will not be presented here but will be discussed hereafter, as they constitute a proof of principle of the efficacy of LV-mediated gene therapy in the correction of WAS T cells defects (Dupre et al., 2006).

4.1.4 LV efficiently transduce WAS PBMC without TCR triggering

Since LV are in principle able to transduce non-dividing cells, transduction of PBMC from patients W1, stimulated by anti-CD3/CD28 mAb or by cytokines was performed in parallel. WAS PBMC were transduced with PGK-GFP (P-G) vector upon short-term culture with IL-2 and IL-7, a pre-stimulation method which allowed efficient *in vitro* transduction of PBMC (Cavalieri et al., 2003). In parallel, PBMC were stimulated through TCR/CD28, in the presence of IL-2 and IL-7 (Figure 6, top). Although the overall transduction efficiency was lower when cytokines alone were used to pre-stimulate WAS PBMC, this kind of stimulation allowed the maintenance of a naïve phenotype, as shown by the expression of CD45RA. As expected, TCR/CD28-driven stimulation allowed high efficiency of transduction (Figure 6, top), but the vast majority of the cells lost the naïve phenotype. We also compared the efficiency of the constitutive PGK promoter and of autologous WAS promoter to transduce WAS PBMC after cytokine mediated stimulation (Figure 6, bottom). A LV in which GFP expression was driven by the *WASP* promoter was used, in parallel with P-G LV. Results indicate that although levels of transduction were lower when the autologous *WASP* promoter was used with respect to the PGK promoter, the *WASP* promoter was able to drive GFP expression in a significant proportion of naïve cells (Figure 6, bottom).

Together, these data indicate that LV are able to transduce efficiently WAS PBMC, while preserving the naïve phenotype. If a gene therapy approach targeting peripheral T cells was envisaged, it would be possible to efficiently transduce T cells without TCR engagement, thus maintaining the natural T cell repertoire and with minimal influence on the homeostasis of transduced cells, which should be re-infused in the patient.

Figure 6

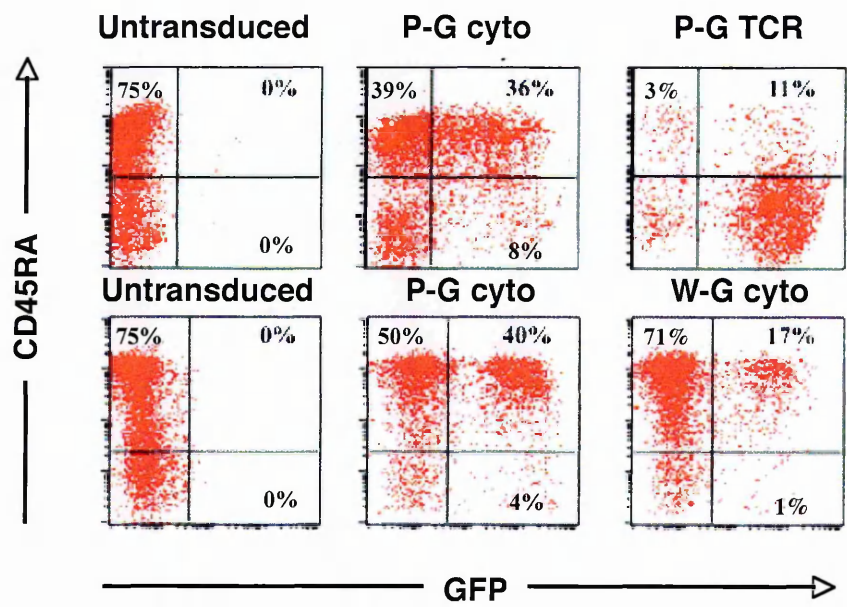


Figure 6. Transduction of PBMC from W1 patient with HIV-derived lentiviral vectors without TCR triggering. PBMC of patients W1 were transduced with p-G LV, after a 48-h prestimulation with anti-CD3 plus anti-CD28 mAb (TCR) or a 96-h prestimulation with IL-2 and IL-7 (cyto). Percentage of transduction (GFP⁺) and of naïve (CD45⁺) cells was analyzed by FACS 10 days after transduction. Two different experiments are shown. Top of the figure shows the comparison between transduction with p-G vector with TCR stimulation (TCR) versus cytokine stimulation (cyto). Bottom of the figure shows comparison of transduction with the constitutive PGK promoter (P-G) and the autologous WASP promoter (W-G) after cytokine stimulation.

4.1.5 Exogenous IL-2 restores proliferative response to anti-CD3 mAb in WAS

CD8⁺ T cell lines

It was been previously shown, both in human T cell lines and in freshly isolated T cells from WASP knock-out mice, that WASP deficiency leads to defective IL-2 secretion (Dupre et al., 2006; Molina et al., 1993; Zhang et al., 1999). Therefore, we investigated whether defective proliferation of WAS CD4⁺ and CD8⁺ T cell lines was due to suboptimal IL-2 production. First, the ability of CD4⁺ (Figure 7A) and CD8⁺ (Figure 7B) T cells from W1 and W2 patients to proliferate in response to different doses of exogenous IL-2 was investigated, showing that WAS T cells respond normally to exogenous IL-2. These data indicate that defective proliferation of WAS T cells in response to anti-CD3 mAb is probably due to lack of sufficient IL-2 secretion. In order to assess whether the addition of exogenous IL-2 was able to rescue the proliferation of WAS T cells in response to TCR triggering, CD4⁺ (Figure 7C) and CD8⁺ (Figure 7D) T cells were stimulated with anti-CD3 mAb-coated beads in the presence of escalating doses of IL-2. Since W1 and W2 CD4⁺ T cells proliferated more than W1 and W2 CD8⁺ T cells in response to TCR triggering, WAS CD4⁺ T cells were stimulated with a lower dose of anti-CD3 mAb (0.5 µg/ml) than CD8⁺ T cells (1 µg/ml), in order to better evaluate the contribution of IL-2 to the proliferation. Addition of IL-2 did not increase the proliferation of control T cells (Figure 7C and 7D), since, at the dose of anti-CD3 mAb used in the experiment, they had already reached the plateau of proliferation. In contrast, addition of IL-2 to anti-CD3 mAb enhanced the proliferation of both WAS CD4⁺ and CD8⁺ T cells. While in WAS CD4⁺ T cells the combination of IL-2 anti-CD3 mAb induced a proliferative response similar to the one induced by IL-2 alone, in WAS CD8⁺ T cells low doses of IL-2 (12.5-25 IU/ml), combined to anti-CD3 mAb, elicited higher proliferation than IL-2 or anti-CD3 mAb alone. Therefore IL-2 and anti-CD3 mAb had a synergistic effect on the proliferation of WAS CD8⁺ T cells.

Together, these data indicate that WAS T cells respond normally to IL-2 and that their defective proliferation in response to anti-CD3 mAb is mainly due to defective IL-2 secretion.

Figure 7

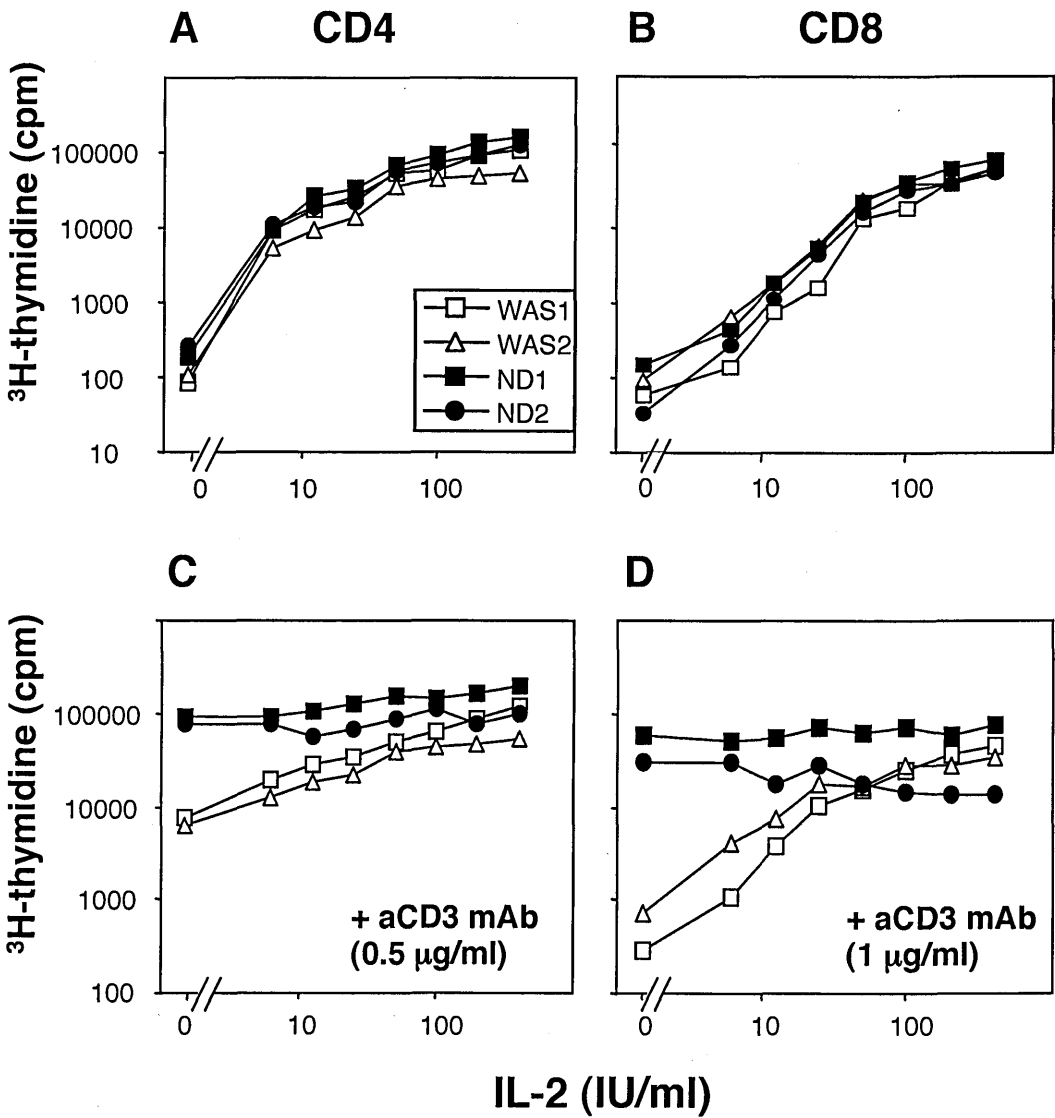


Figure 7. Proliferation of WAS T cell lines in response to exogenous IL-2. Proliferation was evaluated by ^3H -thymidine incorporation in CD4^+ (A) and CD8^+ (B) T cell lines from patients W1 and W2 and from two healthy donors, in response to escalating doses of IL-2. Alternatively, CD4^+ (C) and CD8^+ (D) T cell lines were stimulated with IL-2 in the presence of a fixed dose of anti-CD3 mAb. Values represent triplicate cultures. One representative experiments out of four is shown.

4.2 WASP is required for effector functions of CD4⁺ and CD8⁺ T cells

4.2.1 Defective production of Th1 cytokines by WAS CD4⁺ T cell lines upon TCR/CD28 triggering

To further analyze the cytokine profile of WAS T cell lines, secretion of different cytokines was analysed upon stimulation of the cells with anti-CD3 plus anti-CD28 mAb-coated beads. As expected, IL-2 secretion was lower than the normal range, but not abolished, in WAS CD4⁺ T cells (Figure 8 $p < 0.05$). Interestingly, patient W2, who had a more severe clinical phenotype than patient W1, showed a more severe impairment in IL-2 secretion than patient W1. IFN- γ and TNF- α secretion was absent or severely compromised in both patients (Figure 8 $p < 0.01$). On the contrary, secretion of IL-4, IL-5 and IL-10 did not differ significantly between patient W1 and control CD4⁺ T cells. In CD4⁺ T cells from patient W2, the secretion of IL-5 was slightly defective (Figure 8 $p < 0.05$), while secretion of both IL-4 and IL-10 was normal (Figure 8). Upon stimulation with TPA/ionomycin, the secretion of all the cytokines tested was comparable among WAS and control CD4⁺ T cells (not shown).

To further confirm the differential Th1 versus Th2 cytokine profile, production of IL-2, IFN- γ and IL-4 was investigated by intracellular staining in CD4⁺ T cells from four WAS and one XLT patients. As shown in Figure 9, in all WAS patients the percentages of CD4⁺ T cells producing IL-2 and/or IFN- γ were strongly reduced, with respect to healthy donor cells stimulated through TCR/CD28 [range of IL-2⁺ cells for healthy donors ($n=5$) was: 28-44%; range of IFN- γ ⁺ cells was: 17-30%]. In particular, in WAS patients the population of cells producing both IL-2 and IFN- γ was the one displaying the strongest reduction. Interestingly, CD4⁺ T cells from the XLT patient produced normal levels of IL-2, while IFN- γ production remained below the normal range. Conversely, stimulation with TPA and ionomycin induced robust production of IL-2 and IFN- γ both in patients and in control cells. Intracellular IL-4 production by

WAS patients CD4⁺ T cells was considerably higher than IL-2 and IFN- γ production (Figure 9). When cells producing IL-4 only were considered, percentages among WAS patients were just below the normal range [range of IL-4⁺ cells for healthy donors (n=5) was:22-38%]. However, the percentage of cells producing both IL-4 and IFN- γ was strongly reduced in WAS CD4⁺ T cells compared to control cells.

Together, these data demonstrate that in CD4⁺ T cells from WAS patients, the secretion of Th1 cytokines is defective, while the secretion of Th2 cytokines is normal, or minimally reduced.

Figure 8

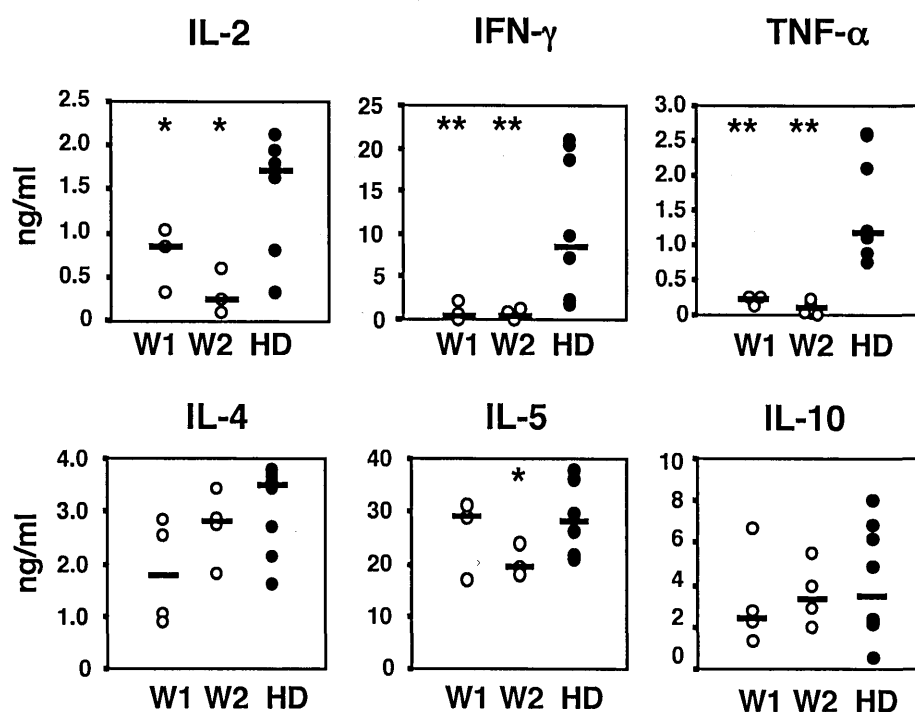


Figure 8. Pattern of cytokines secretion by WASP-deficient CD4⁺ T cell lines. Secretion of IL-2, IFN- γ , TNF- α , IL-4, IL-5, and IL-10 was evaluated by capture ELISA performed on the supernatants of CD4⁺ T cell lines from patients W1 and W2 and two healthy donors stimulated with beads coated anti-CD3 (1 μ g/ml) and anti-CD28 (10 μ g/ml). Values represent the mean of triplicate cultures. Three to four independent experiments are shown in each graph. Values from two healthy donors were shown for each experiment. Background levels were subtracted. Statistical analysis was performed using the Wilcoxon Signed Rank Test for IL-2 and the Wilcoxon Rank Sum Test for the other cytokines. Significance is represented as follows: * p<0,5; ** p<0,01.

Figure 9

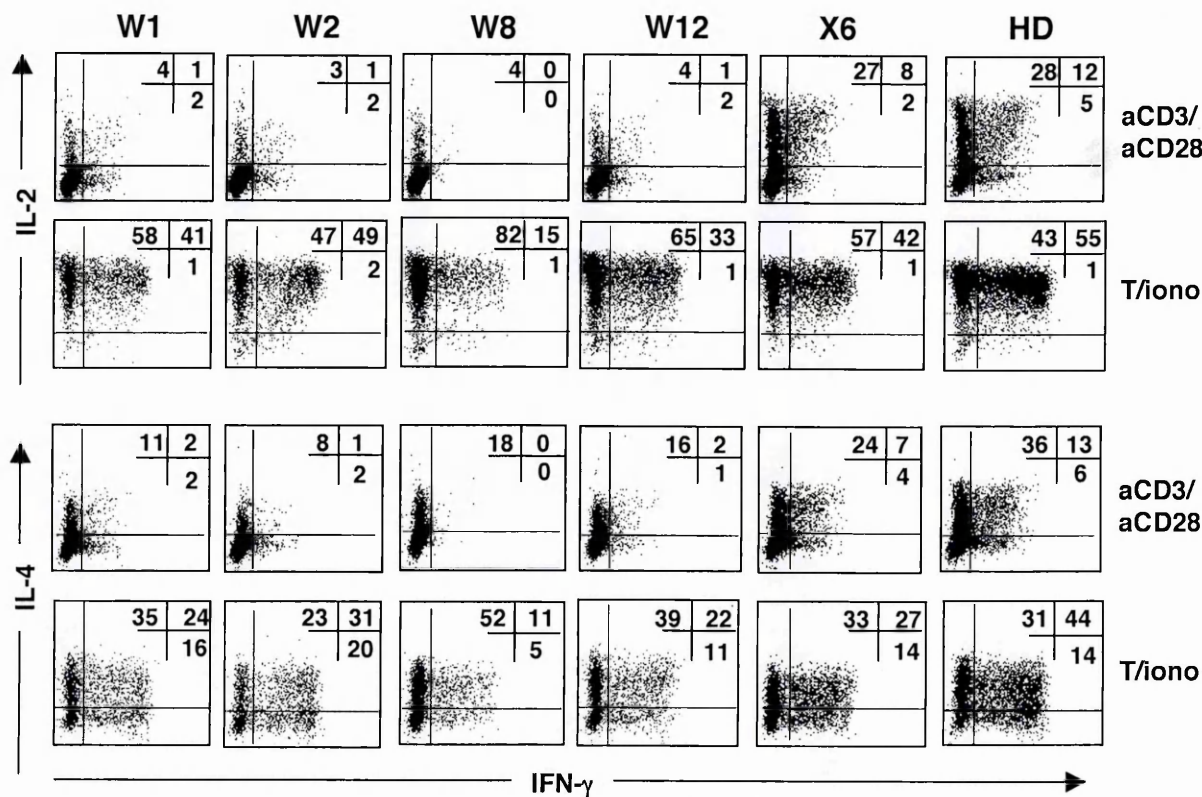


Figure 9 . Pattern of cytokines production by WASP-deficient CD4⁺ T cell lines. CD4⁺ T cell lines from WAS patients and healthy donors were stimulated with beads coated with 1 µg/ml anti CD3 plus 10 µg/ml anti-CD28 mAb, or with TPA plus ionomycin. Production of IFN-γ versus either IL-2 or IL-4 was analyzed by intracytoplasmic staining. Only percentages ≥1 are indicated. Experiment shown is representative of three independent experiments including all the patients and of six experiments including patients W1 and W2. One representative healthy donor out of five is shown. Ranges of cytokine production by T cells from the five healthy donors is indicated in the text.

4.2.2 Defective TCR/CD28-mediated IL-2 and IFN- γ production by WAS T cells cultured in Th1-polarizing conditions

To determine whether the failure to produce Th1 cytokines was due to the inability of WAS naïve CD4⁺ T cells to differentiate into Th1 cells, CD45RO⁻/CD4⁺ T cells from patient W1 were cultured in Th1-polarizing conditions and tested for cytokine production after one round of stimulation. To overcome differences in IL-2 levels secreted by WAS and control cell cultures, differentiation was carried out in the presence of exogenous IL-2. Upon stimulation with anti-CD3 plus anti-CD28 mAb, the percentage of IFN- γ -producing cells was higher both in W1 and control cells grown in Th1-polarizing conditions, with respect to the same cells cultured in Th0 conditions (Figure 10). Furthermore, IL-4 production was down-regulated (not shown), indicating effective Th1 differentiation. However, upon TCR/CD28-mediated stimulation, W1 cells showed a strong reduction in the proportion of cells producing IFN- γ , with respect to control cells. The defect was mostly evident in the subset of IL-2⁺/IFN- γ ⁺ CD4⁺ T cells. On the contrary, stimulation with TPA/ionomycin induced similar levels of cytokine production in W1 and control Th1 cells, indicating that these cells are intrinsically able to produce IL-2 and IFN- γ .

These data show that, although WASP-deficient CD4⁺ T cells can undergo Th1 differentiation in culture conditions in which IL-12 is supplied, they retain a defect in the production of IFN- γ and IL-2 in response to TCR/CD28-mediated signals.

Figure 10

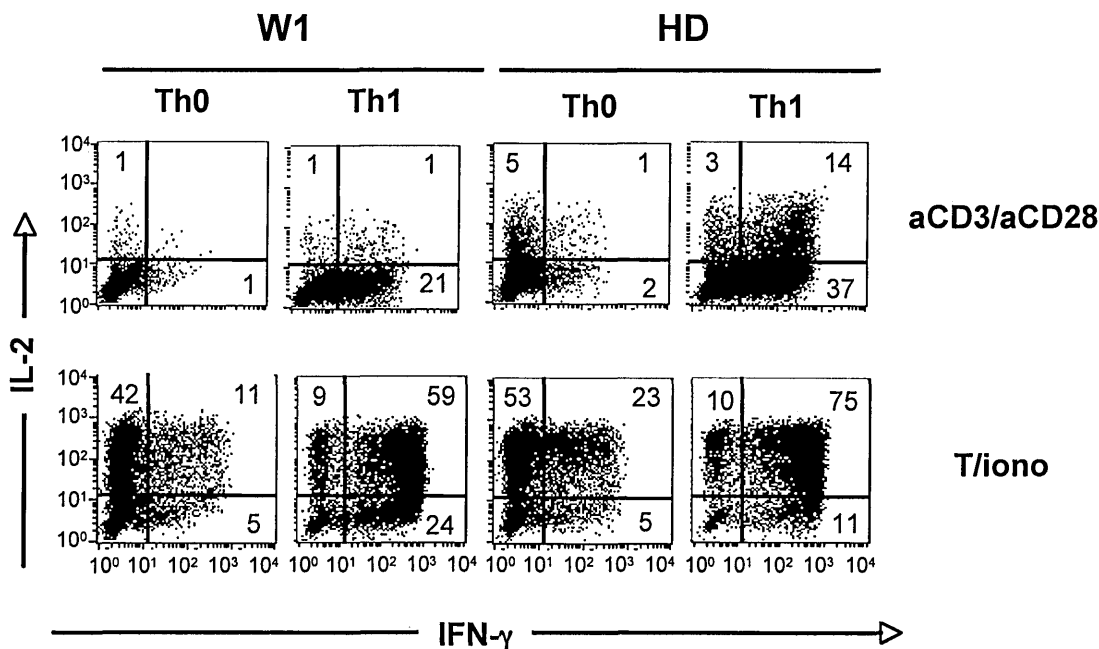


Figure 10. Th1 polarization in WAS CD4⁺ T cells. Intracytoplasmic staining of cytokine production by Th0 and Th1 cells generated from naïve CD4⁺ T cells of WAS1 and one normal donor (out of two) is shown. Cells cultured for one week in either Th0 or Th1-polarizing conditions were stimulated for 6 hr with either 10 µg/ml immobilized anti-CD3 plus 1 µg/ml soluble anti-CD28 mAbs or TPA plus ionomycin, in the presence of brefeldin A. Only percentages ≥1 are indicated. One representative polarization experiment out of two is shown. Values of cytokine production were confirmed in three independent stainings.

4.2.3 Defective cytokine production by WAS CD8⁺ T cell lines

In order to investigate the consequences of WASP deficiency in CD8⁺ T cells, cytokine secretion and production by WAS and XLT CD8⁺ T cell lines, generated in parallel to the CD4⁺ T cell lines described above, was investigated. CD8⁺ T cells from patients W1 and W2 showed a complete block in the secretion of IL-2, IFN- γ and TNF- α (Figure 11) ($p < 0,01$ for the three cytokines tested). IL-5 secretion by WAS CD8⁺ T cells was also reduced compared to control CD8⁺ T cells (range observed in three independent experiments was: 0.2-0.7 ng/ml IL-5 in WAS CD8⁺ T cells; 3.2-22.6 ng/ml IL-5 in control CD8⁺ T cells) (not shown). IL-4 and IL-10 were low or undetectable (0-400 pg/ml) in the supernatants of both WAS and control CD8⁺ T cells (not shown).

Figure 11

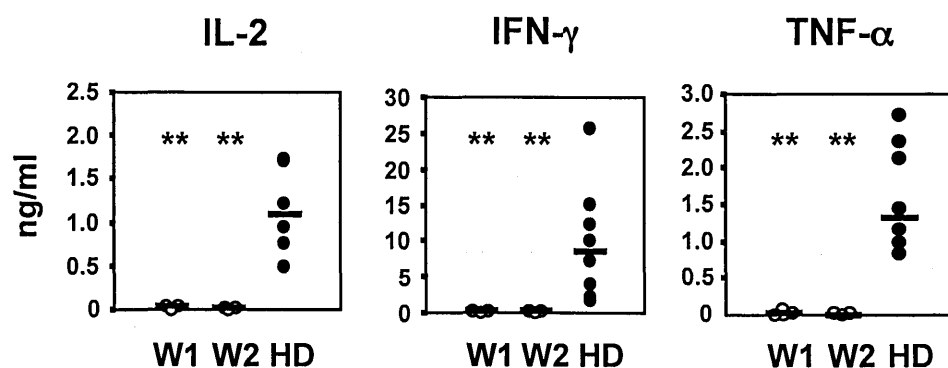


Figure 11. Pattern of cytokines secretion by WASP-deficient CD8⁺ T cell lines. Secretion of IL-2, IFN- γ , TNF- α was evaluated by capture ELISA performed on the supernatants of CD8⁺ T cell lines from patients W1 and W2 and two healthy donors stimulated with beads coated with 1 μ g/ml anti-CD3 plus 10 μ g/ml anti-CD28. Values represent the mean of triplicate cultures. Three to four independent experiments are shown in each graph. Values from two healthy donors were shown for each experiment. Background levels were subtracted. Statistical analysis was performed using the Wilcoxon Rank Sum Test. Significance is represented as follows: * $p < 0,5$; ** $p < 0,01$.

According with the results of cytokine secretion, a strong reduction in the proportion of cells producing IL-2 and IFN- γ in response to TCR and CD28 triggering was observed by intracellular staining in all WAS patients (Figure 12) [range of IL-2⁺ cells in healthy donors (n=5) was: 29-47%; range of IFN- γ ⁺ cells was: 26-65%]. Similarly to what shown for CD4⁺ T cells, CD8⁺ T cells from the XLT patient displayed normal production of IL-2 and sub-optimal IFN- γ production. In different experiments, levels of IL-2 and IFN- γ production by CD8⁺ T cells from this patient were either within or slightly below normal range, and consistently higher than those found in WAS CD8⁺ T cells. Although stimulation with TPA/ionomycin induced robust production of IL-2 and IFN- γ by CD8⁺ T cells from all patients (Figure 12), in patients W2, W8 and W12, a reduction in cells producing both IL-2 and IFN- γ was still present [range of IL-2⁺/ IFN- γ ⁺ cells in healthy donors (n=5) was: 75-89%].

Together, these results show an inability of CD8⁺ T cells from WAS patients to produce and consequently secrete IL-2, IFN- γ and TNF- α in response to TCR/CD28 triggering.

Figure 12

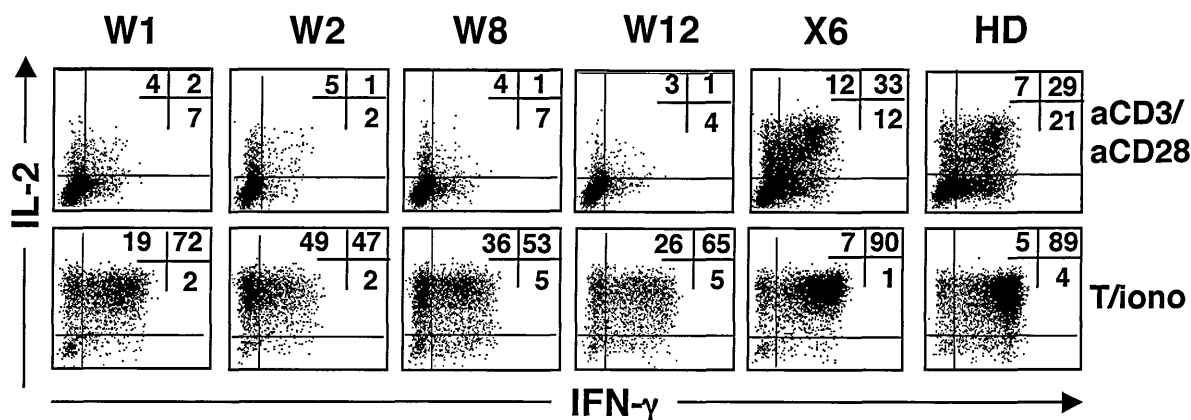


Figure 12. Pattern of cytokines production by WASP-deficient CD8⁺ T cell lines. CD8⁺ T cell lines from WAS patients and healthy donors were stimulated with beads coated with 1 μ g/ml anti-CD3 plus 10 μ g/ml anti-CD28 with or with TPA plus ionomycin. Production of IFN- γ versus IL-2 was analyzed by intracytoplasmic staining. Only percentages ≥ 1 are indicated. Experiment shown is representative of three independent experiments including all the patients and of six experiments including patients W1 and W2. One representative healthy donor out of five is shown. Ranges of cytokine production by T cells from the five healthy donors is indicated in the text.

4.2.4 Defective cytokine production by PBMC from WAS patients

It is important to determine the *in vivo* relevance of our findings regarding the profile of cytokine production by WAS T cell lines. In particular, it would be important to verify whether a different ability to produce Th1 versus Th2 cytokines by Th cells is present also in freshly isolated T cells. To this aim, cytokine secretion by total PBMCs isolated from two WAS patients (W11 and W13) was analyzed upon stimulation with anti-CD3/CD28 mAb or with TPA/ionomycin. The pattern of cytokine secretion is depicted in Figure 13. In accordance to what observed in WAS T cell lines, the secretion of IL-2, IFN- γ and TNF- α was reduced in PBMC from both WAS patients, compared to control cells, after stimulation with anti-CD3 plus anti-CD28 mAb. Stimulation with TPA plus ionomycin induced normal production of IL-2, IFN- γ and TNF- α in PBMC from patients W11, while production of both IL-2 and TNF- α by W13 PBMC was reduced with respect to control cells, also upon TPA/ionomycin stimulation. These data show that IL-2 and IFN- γ are not secreted by WAS PBMC upon TCR mediated activation and therefore confirm our data obtained in T cell lines.

We noted that levels of IL-4 and IL-5 secreted by primary T cells from normal donors after TCR/CD28 triggering are much lower than those secreted by CD4⁺ T cell lines. IL-4 secretion by WAS PBMC was defective both upon TCR/CD28 triggering and TPA/ionomycin stimulation. IL-5 secretion was defective in W13 PBMC and in the low range of normal values in W11 PBMC, upon TCR/CD28 triggering. Stimulation with TPA plus ionomycin induced similar levels of IL-5 secretion in WAS and control PBMC.

It should be noted that patient W11 was under steroids treatment, at the time of blood sampling, which could have negatively influenced the secretion of cytokines by his cells. Analysis of cytokine production profile is being currently extended to other

WAS patients, with different clinical phenotypes. However, this analysis depends on several variables, first of all the effective proportion of CD4⁺ and CD8⁺ T cells among total PBMC. Extension of this analysis to PBMC of other WAS patients will be essential to confirm that a dichotomy of Th1 versus Th2 cytokine production is already present in freshly isolated cells.

Figure 13

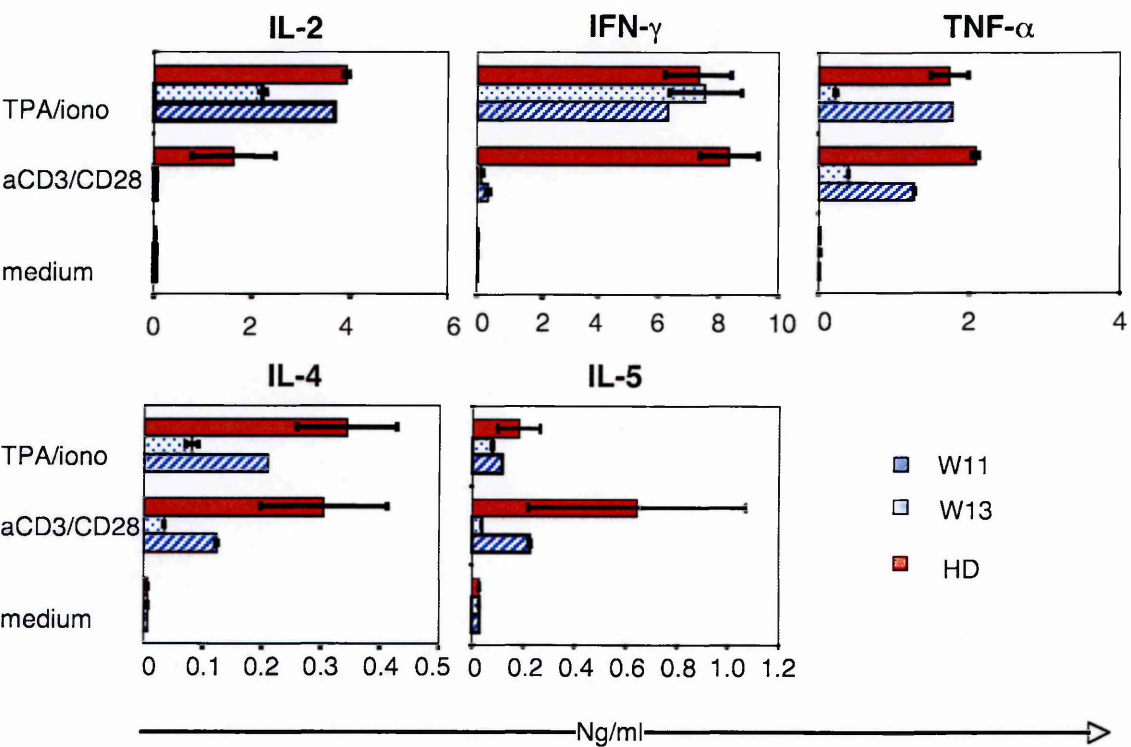


Figure 13. Pattern of cytokines production by freshly isolated PBMC from two WAS patients. Freshly isolated PBMC from patients W11 and W13 and from four healthy donors (HD) were stimulated with immobilized anti-CD3 plus soluble anti-CD28 mAb or with TPA plus ionomycin. Secretion of cytokines was evaluated after 18 hours (IL-2) or 48 hours (all the other cytokines). Error bars represent standard deviation.

4.2.5 Normal lytic activity of WAS CD8⁺ T cells against allogeneic cells

Previous studies showed that NK cells isolated from WAS patients displayed reduced lytic activity (Gismondi et al., 2004), which could be restored by the addition of IL-2. No data were available regarding the effect of WASP deficiency on the lytic activity of CTL. Therefore, CD8⁺ T cell lines specific for an allogenic B-EBV cell line (JY) were generated starting from PBMC of patient W1 and of two healthy donors and analyzed for their ability to lyse JY cells. As shown in Figure 14A, the percentage of lysis of target cells was comparable in W1 and control CD8⁺ T cells at the different effector:target ratio tested. Exhaustion of the NK-like lytic activity, which could partially account for the lysis of JY cells, by pre-incubation with the MHC class I-negative K562 cell line, resulted in a reduction of the JY-specific lytic activity especially by W1 CD8⁺ T cells, which nevertheless remained within the normal range (Figure 14B). EBV-transformed B cells are known to be very strong stimulators, as they express high levels of costimulatory molecules. In order to investigate whether WAS CTLs could lyse efficiently also non-transformed allogenic cells, three different allo-specific CD8⁺ T cell lines were established from PBMC of patient W1 and of three healthy donors. Lytic activity was then tested using PHA-blasts, according to the cell line specificity. WAS CTL were able to lyse target cells as efficiently as control cells also in this system (Figure 14C).

These data suggest that WASP-deficiency does not affect the ability of CTL to lyse allogenic cells. Therefore, WASP seems to be dispensable for lytic function, although it is required for T cell activation and the production of effector cytokines. However, in order to generalize this concept, the Ag-specific activity of WAS CTL needs to be investigated and the analysis needs to be extended to other patients.

Figure 14

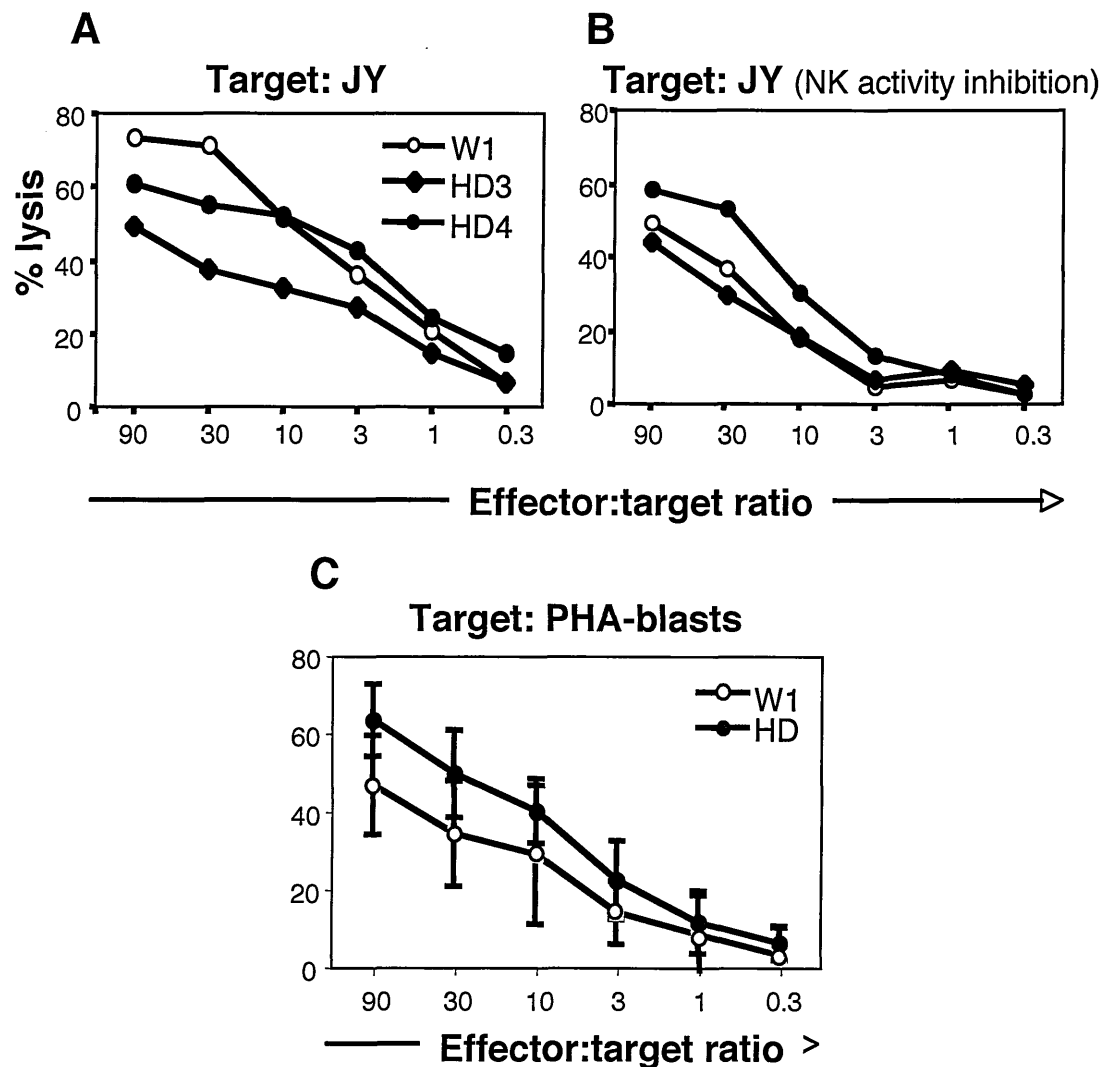


Figure 14. Lytic activity of CD8⁺ T cell lines from patient W1. Cytotoxic activity of JY-specific CD8⁺ T cells from patients W1 and two healthy donors directed against allogeneic EBV-transformed B cells (JY), in the absence (A) or in the presence (B) of non-radiolabeled K562 cells (cold inhibition of NK-like lytic activity) is shown. Each point of the indicated effector:target ratio is the mean of duplicate values. One representative experiment out of three is shown. Cytotoxic activity of JY/allogeneic donor-specific CD8⁺ T cells from patients W1 and from healthy donors against allogeneic PHA-blasts (C). Results represent the average of the specific lysis by three different cell lines from patient W1 and three different cell lines, with the same specificity, derived from three different healthy donors. Error bars represent standard deviation.

4.3 WASP is required for T cell activation and effector functions: cellular and molecular mechanisms

4.3.1 WASP is recruited to detergent-resistant membranes (DRM) upon T cell activation

Previous findings indicated that WASP is recruited at the site of contact between T cells and APC and is required for IS formation (Badour et al., 2003; Cannon and Burkhardt, 2004; Cannon et al., 2001; Krause et al., 2000). Since IS has been shown to be accompanied by the re-organization and clustering of lipid rafts and re-orientation of actin cytoskeleton, we decided to study the sub-cellular localization of WASP during T cell activation, investigating in particular whether WASP could ever be recovered into DRM fractions. Therefore, resting T cell lines from healthy donors were stimulated with anti-CD3 plus anti-CD28 mAbs and detergent-resistant membranes (DRM), which have been proposed to represent the counterpart of lipid rafts in intact cells (Melkonian et al., 1999), were isolated by flotation in a sucrose gradient, after cell lysis in non-ionic detergent (Simons and Ikonen, 1997). Resting T cell lines from healthy donors were stimulated for the indicated times with anti-CD3 plus anti-CD28 mAb and high-density (non-DRM) and low-density (DRM) fractions were isolated (Figure 15). The localization of LAT, which is constitutively rafts-associated, was then investigated. As shown in Figure 15, the adaptor molecule LAT was always recovered in low-density fractions (DRM), indicating that DRM are enriched for proteins which have a high affinity for rafts in living cells. WASP was found in non-DRM fractions in resting T cell lines (Figure 15). However, already 10 seconds after TCR/CD28 triggering, and at all the other time points of stimulation evaluated, a fraction of WASP was recovered in DRM fractions (Figure 15). CD3- ζ was recovered only in soluble, non-DRM fractions

in resting T cells, while it was recovered into DRM fractions upon T cell stimulation through TCR/CD28, as previously shown (Figure 15) (Montixi et al., 1998; Xavier et al., 1998), with a kinetic that resembled the one of WASP recruitment. These findings also indicate that our fractionation procedure was able to separate membrane proteins according to their affinity with portions of the membrane with different physical characteristics.

Together, these data show that TCR/CD28 triggering in T cells induces the partition of a fraction of WASP to DRM fractions, indicating a change in the physical properties of this protein, which is likely to depend on its re-localization from the cytosol to specific portion of the membrane. Since WASP has been shown to localize at the level of the IS (Cannon et al., 2001) and, in our assay, DRM are highly enriched for a raft-resident protein (LAT), it is possible that WASP re-localization occurs at specific site of plasma membrane corresponding to a subset of lipid rafts.

Figure 15

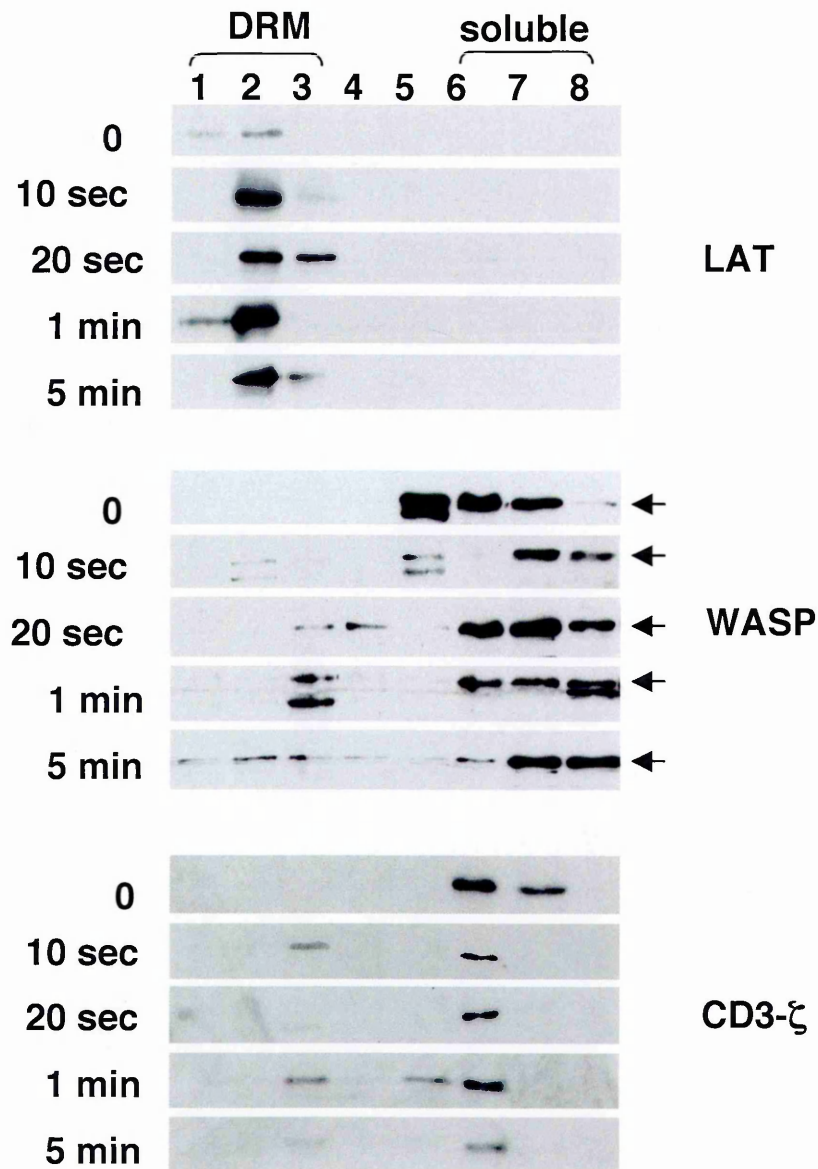


Figure 15. Partition of LAT, WASP and CD3- ζ into DRM and non-DRM (soluble) fractions. Resting T cell line from healthy donor was stimulated through TCR and CD28 for the indicated times. Following lysis with non-ionic detergent and flotation in a sucrose gradient, the different fractions were resolved by SDS-PAGE. The presence of LAT, WASP and CD3- ζ in the different fractions was revealed by hybridization with specific Abs.

4.3.2 Defective GM1 upregulation upon TCR/CD28 triggering in WAS T cell lines

T cell activation has been associated both to clustering of lipid rafts and to elevation of the levels of GM1, a ganglioside enriched in a subset of membrane rafts, on T cell plasma membrane (Lanzavecchia and Sallusto, 2000; Viola et al., 1999). We therefore investigated the levels of GM1 in WAS and control T cell lines, after a three days-stimulation with beads coated with anti-CD3 alone, or plus anti-CD28 mAb (Figure 16A and 16B). In resting T cells from patients W1 and W2 the levels of GM1 were slightly lower than in control T cell lines (Figure 16A and 16B) ($p < 0.001$ t test, in five experiments), while GM1 levels in resting W3 T cells were in the normal range (Figure 16A and 16B). Upon stimulation with beads coated with high dose of anti-CD3 mAbs (10 $\mu\text{g/ml}$) both WAS and control T cell lines up-regulated GM1 on the cell surface, although the final GM1 levels were lower in WAS than in control T cell lines (Figure 16A). Addition of anti-CD28 to anti-CD3 mAb increased the upregulation of GM1 in control T cells at low anti-CD3 mAb doses (Figure 16B), indicating that upregulation of GM1 strongly depends on costimulation in conditions of suboptimal TCR triggering. In T cells from WAS patients the levels of surface GM1 after TCR/CD28 triggering were lower than in control cells (Figure 16B $p < 0.001$ t test, in five experiments at the maximal anti-CD3 mAb dose).

These data suggest that low levels of GM1 on the plasma membrane of WAS T cell lines could be partially responsible for WAS T cell unresponsiveness to TCR/CD28 stimulation. To further investigate whether reduced levels of GM1 on the plasma membrane of WAS T cells were due to a reduction in the total amount of cellular GM1 (intracellular and surface one) or to defective transport of pre-existing GM1 to the plasma membrane, the levels of surface *versus* total GM1 were evaluated in the same stimulation conditions (Figure 16C and 16D). Upon stimulation with either anti-CD3

mAb alone, or anti-CD3 plus anti-CD28 mAb the levels of total GM1 in WAS T cells were lower than in control T cells (Figure 16C and 16D). It should be noted that since the basal GM1 levels were also lower in WAS than in control T cells, the fold increase was not so much different between the two cell populations (WAS: 2.2, HD: 2.5 fold with anti-CD3 mAb; WAS 2.1, HD: 3.1 fold with anti-CD3/CD28 mAb). However, after stimulation, the mean fluorescence intensity (MFI) of surface GM1 was comparable to that of total GM1 in control cells (Figure 16C and 16D), suggesting that the vast majority of the cellular GM1 was on the plasma membrane. On the contrary, in stimulated WAS T cells there were no changes in the MFI of surface *versus* total GM1, when compared to resting WAS T cells, thus suggesting a possible impairment in the transport of GM1 to the plasma membrane.

Figure 16

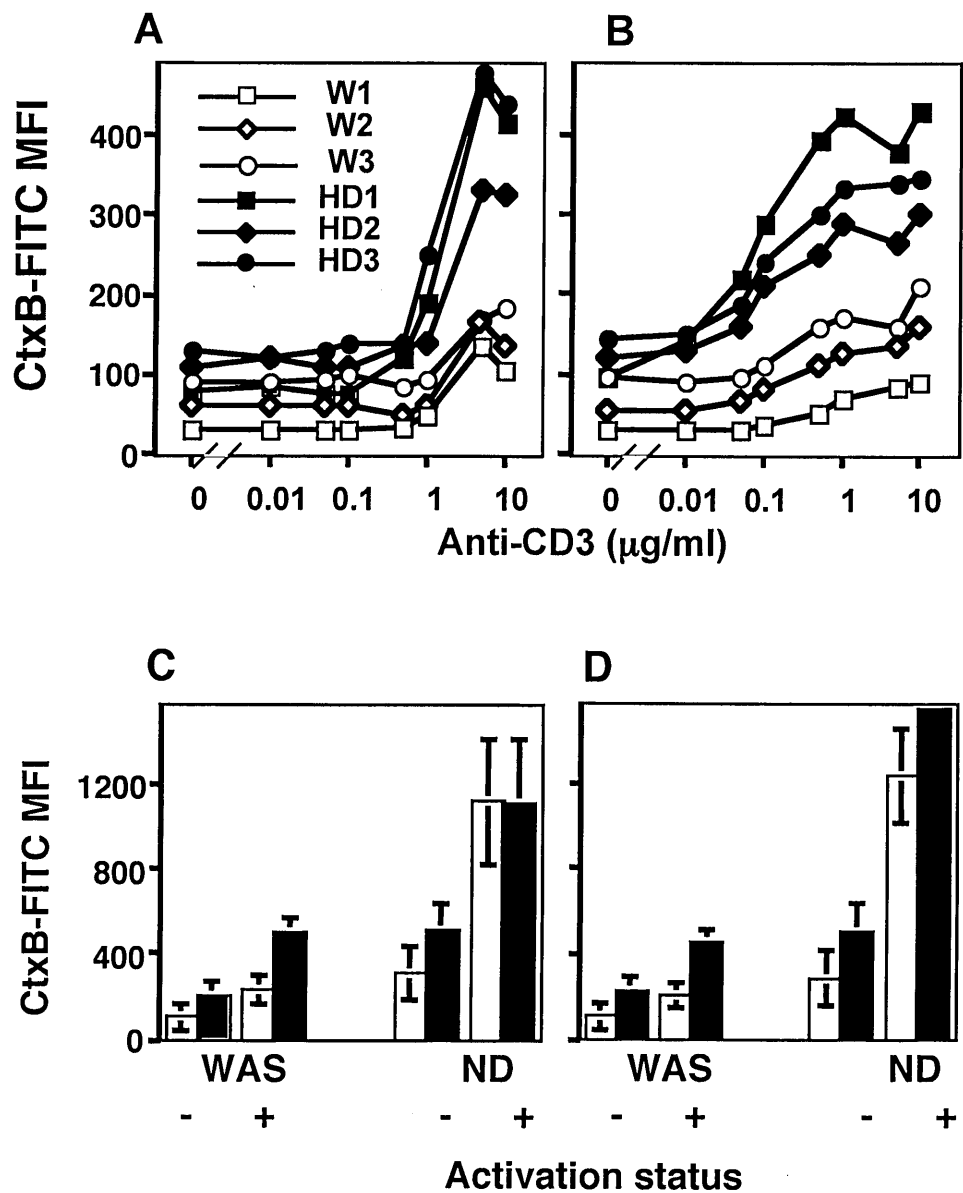


Figure 16. Analysis of GM1 levels on the surface of WAS T cell lines upon TCR/CD28 triggering. T cell lines from patients W1, W2, W3 and from three healthy donors were stimulated for 72 hours with beads coated with the indicated amounts of anti-CD3 alone (A) or anti-CD3 plus 10 $\mu\text{g/ml}$ anti-CD28 mAbs (B). GM1 levels were analyzed by surface staining of the cells with CtxB-FITC. One representative experiments out of three is shown. Parallel analysis of total (black bars) cellular *versus* surface (white bars) GM1 was performed in WAS and control T cell lines stimulated with anti-CD3 mAb only (C) or with anti-CD3 plus anti-CD28 mAb (D).

In order to evaluate the contribution of CD4⁺ versus CD8⁺ T cells to the defects in GM1 upregulation observed in WAS T cells, the analysis of surface GM1 levels was performed in purified T cell subsets from patients W1 and W2 and from two healthy donors (Figure 17). Stimulation of control T cells with beads coated with escalating doses of anti-CD3 mAb induced the upregulation of GM1 on the cell surface, at higher levels than in WAS T cells (Figure 17). The impairment of GM1 upregulation was more profound in CD8⁺ than in CD4⁺ T cells from these WAS patients.

Figure 17

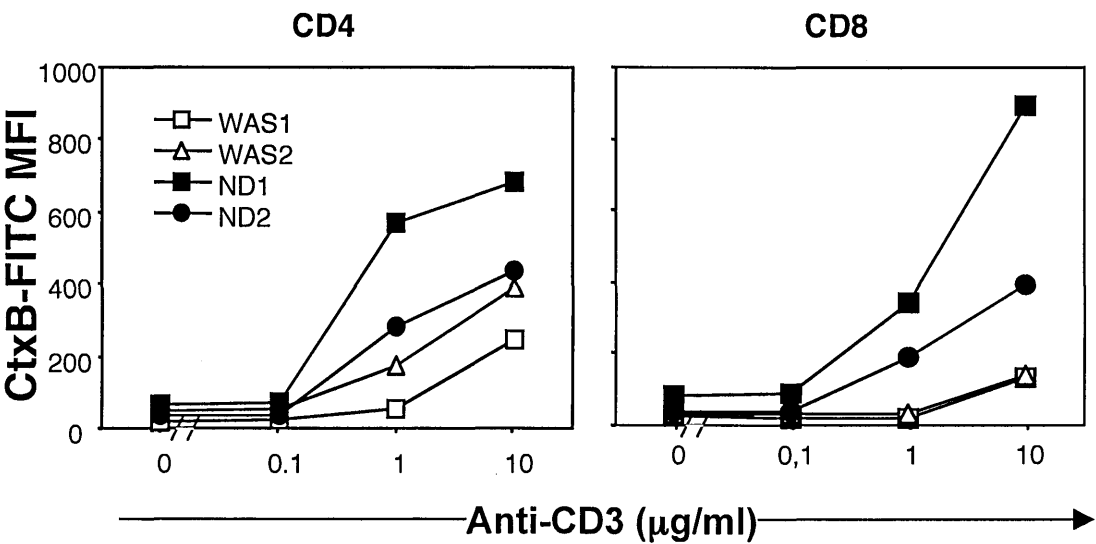


Figure 17. Analysis of GM1 levels on the surface of WAS T cell lines upon TCR triggering. CD4⁺ (A) and CD8⁺ (B) T cell lines from patients W1, W2 and from two healthy donors were stimulated for 72 hours with beads coated with the indicated amounts of anti-CD3 alone. GM1 levels were analyzed by surface staining of the cells with CtxB-FITC. One representative experiments out of three is shown.

Together, these data indicate that defective activation of WAS T cells correlates with defective GM1 upregulation, which reflects a reduction in both the total amount of cellular GM1 and in the relative levels of GM1 on the plasma membrane. Although basic organization of rafts in WAS T cells seems not to be substantially altered in steady-state, a more detailed analysis of rafts composition both in terms of lipids and associated proteins will clarify if and to what extent lipid rafts organization could be affected by WASP deficiency.

4.3.1 Inhibition of glycosphingolipids synthesis in T cell lines does not affect either proliferation or IL-2 secretion

To investigate the functional consequences of reduced levels of GM1 on T cell activation, the synthesis of glycosphingolipids was inhibited in normal T cell lines, during stimulation through TCR/CD28, to reproduce the situation observed in WAS T cell lines. To this aim, T cells from healthy donors were stimulated with plastic-bound anti-CD3 plus or minus anti-CD28 mAb, in the presence of the ceramide analogue PDMP, which inhibits the synthesis of glucosyl-ceramide, and consequently of glycosphingolipids. Analysis of GM1 levels on plasma membrane of resting and activated T cells showed that a non-toxic dose of PDMP (10 μ M) blocked GM1 upregulation, but not the increase of cell size, in T cells stimulated with immobilized anti-CD3 plus anti-CD28 mAbs (Figure 19A). Quantification of GM1^{bright} cell population in T cells, after 72 hours of stimulation with anti-CD3 mAb alone or anti-CD3 plus anti-CD28 mAb showed that PDMP prevented the upregulation of GM1 (Figure 19B $p < 0.05$ for GM1 fold increase in untreated, activated cells, *versus* PDMP-treated cells). Addition of PDMP did not have significant effect on unstimulated T cells

(not shown), which was probably due to the relatively slow turnover of glycosphingolipids in resting cells. However, the rate of cell division, as assessed by CFSE dilution after 72 hours of stimulation, was not significantly affected by PDMP treatment (Figure 19C). IL-2 secretion was also analyzed, showing that PDMP treatment induced a reduction in IL-2 secreted by T cells stimulated with anti-CD3 mAb alone (Figure 19D $p<0.05$). However, the overall levels of IL-2 secreted by T cells stimulated through TCR alone were very low. Addition of anti-CD28 to anti-CD3 mAb induced the secretion of higher levels of IL-2, which was not significantly affected by PDMP treatment (Figure 19D).

Figure 19

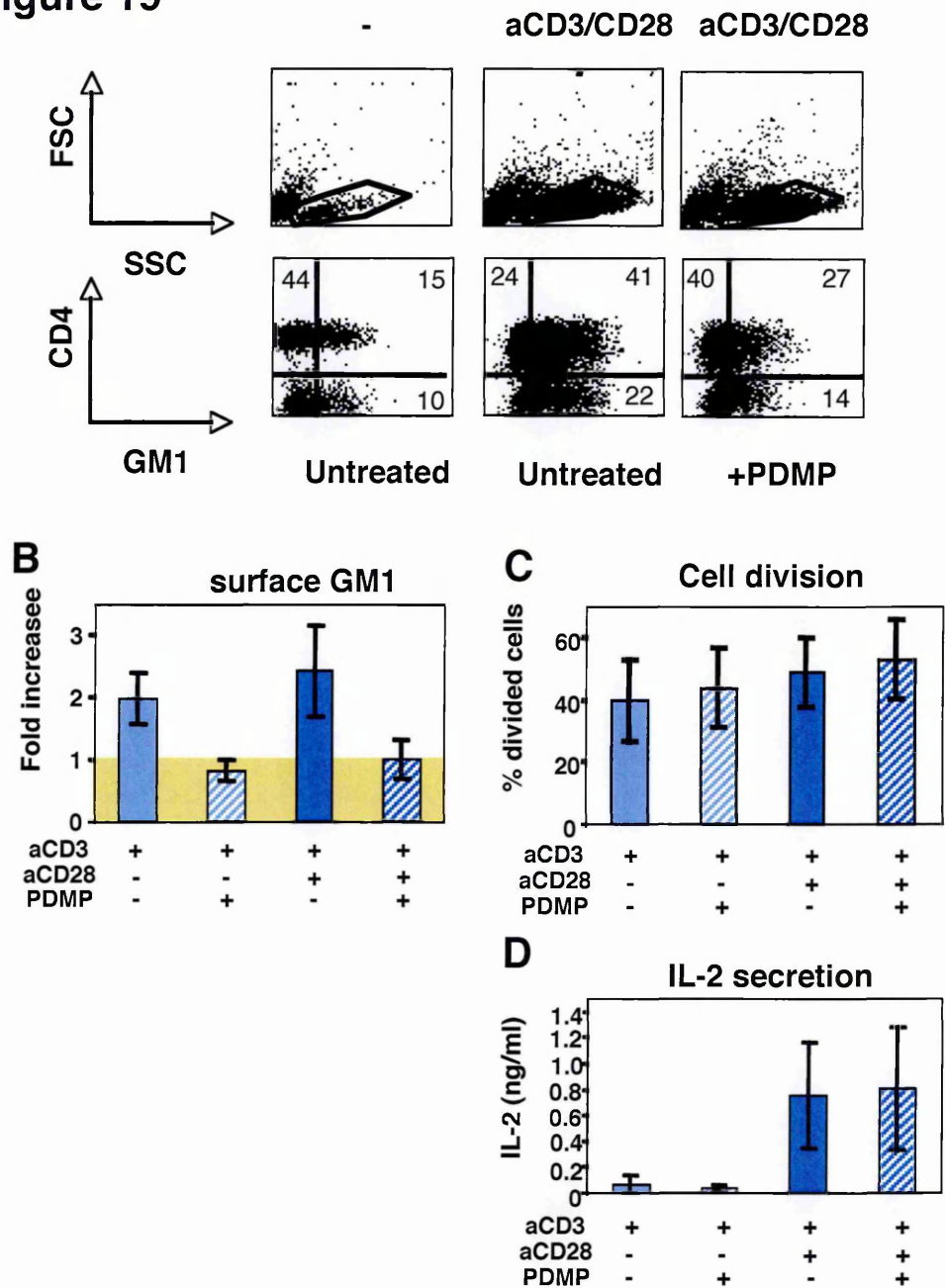


Figure 19. Effect of inhibition of glycosphingolipids synthesis on T cell activation. T cell lines from healthy donors were loaded with CFSE and stimulated with imm.aCD3 plus soluble anti-CD28 mAb, in the presence, or absence, of the glycosphingolipid synthesis inhibitor PDMP. After 72 hours, levels of surface GM1 were evaluated by CtxB-FITC staining (A). Quantification of GM1 fold increase was evaluated considering the percentages of GM1 positive cells in T cells from healthy donors (n=6) (B). Proliferation was evaluated by analysis of CFSE dilution at day three (C) and IL-2 secretion was measured in the supernatants collected after 18 hours of stimulation (D).

These experiments were performed by stimulating the cells with immobilized anti-CD3 mAb. It is possible that the overall independence of T cell activation on glycosphingolipids levels could be due to the fact that formation of IS-like structure is not required for optimal T cell activation, in this system. Therefore, the effect of PDMP treatment during stimulation with beads coated with anti-CD3 alone or anti-CD3 plus anti-CD28 mAb was also evaluated. Similarly to what shown here before, GM1 upregulation was completely prevented by the addition of PDMP (Figure 20A and 20B), although the overall upregulation of GM1 induced by Ab-coated beads was lower than the one induced by immobilized anti-CD3 plus soluble anti-CD28 mAb. In addition, we found that TCR/CD28-mediated stimulation induced also the upregulation of another ganglioside, GM3 (Figure 20A). GM3 was expressed at higher levels than GM1, indeed all resting T cells were GM3⁺. GM3 upregulation was also prevented by PDMP (Figure 20A). Proliferation of T cells in response to anti-CD3 or anti-CD3/CD28 mAb-coated beads was not significantly affected by PDMP treatment (Figure 20C). Accordingly, IL-2 secretion elicited by TCR/CD28 triggering was comparable in untreated and PDMP-treated cells (Figure 20D). Stimulation with beads coated with anti-CD3 mAb alone induced very low levels of IL-2 (Figure 20D).

Altogether, these results indicate that prevention of glycosphingolipids upregulation on the plasma membrane during T cell activation does not block proliferation or IL-2 secretion. Therefore, reduced glycosphingolipids levels on stimulated WAS T cells are likely to be the consequence and not the reason for WAS T cells unresponsiveness to TCR/CD28-driven signals. In WAS T cells, defective TCR/CD28 responsiveness could be due to impaired formation of the IS, which affects several “late” events of T cell activation, including upregulation of plasma membrane glycosphingolipids.

Figure 20

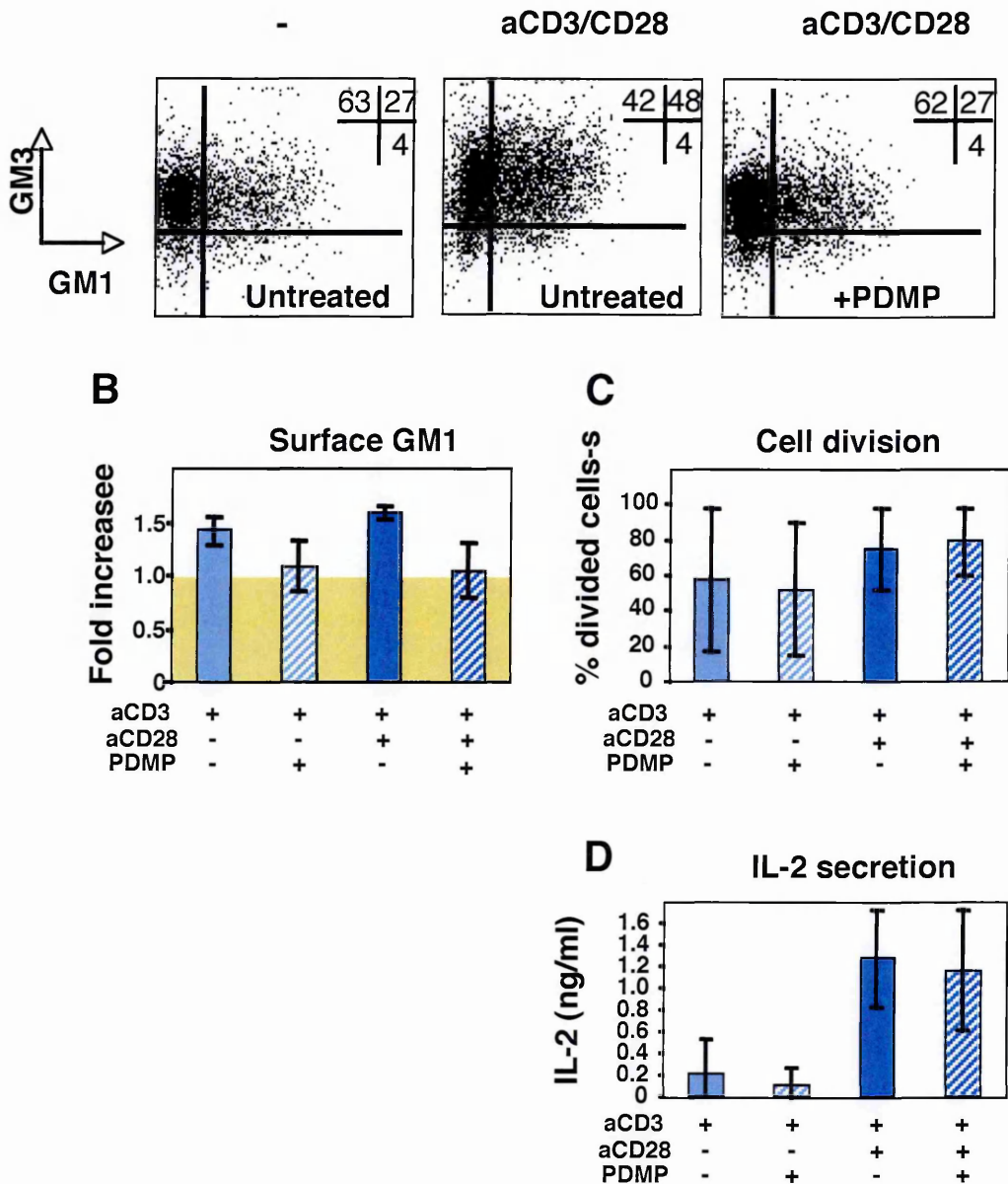


Figure 20. Effect of inhibition of glycosphingolipids synthesis on T cell activation. T cell lines from healthy donors were loaded with CFSE and stimulated with beads coated with anti-CD3 plus anti-CD28 mAb, in the presence, or absence, of the glycosphingolipid synthesis inhibitor PDMP. After 72 hours, levels of surface GM1 was evaluated by CtxB-FITC staining (A). Quantification of GM1 fold increase was evaluated considering the percentages of GM1 positive cells in T cells from healthy donors (n=4) (B). Proliferation was evaluated by analysis of CFSE dilution at day three (C) and IL-2 secretion was measured in the supernatants collected after 18 hours of stimulation (D).

4.3.4 WAS T cells display normal ability to adhere to immobilized anti-CD3 mAb or fibronectin

Reduced proliferation in response to anti-CD3 mAb stimulation could be also due to an inability of WAS T cells to adhere to a surface coated with anti-CD3 mAb. Therefore, we investigated the adhesion of T cell lines from patients W1 and W2 and from three different healthy donors to plastic-bound anti-CD3 mAb coated at different doses. Stimulation with the lowest anti-CD3 mAb dose (0.1 $\mu\text{g/ml}$) elicited in some cases an increase of the adhesion of control T cells, but never of WAS T cells (Figure 21A). However, this difference was not significant. At the other doses of anti-CD3 mAb tested, WAS and control cells showed comparable adhesion (Figure 21A). Formation of conjugates between T cells and APC involves initially the interaction of adhesive molecules, such as integrins. If the TCR is productively engaged, the avidity of integrins binding to their ligands is positively modulated by a mechanism called inside-outside signalling (Kinashi, 2005). We therefore analyzed the ability of WAS and control T cells to adhere to fibronectin (a natural ligand of integrins), in the presence of the same doses of anti-CD3 mAb tested before. Figure 21B shows that adhesion of WAS and control T cells to fibronectin alone was low, while addition of anti-CD3 mAb induced an increase in the adhesive capacity of both WAS and control cells. Similarly to what was shown for the binding to anti-CD3 mAb only, a reduction in the adhesion capacity, although not statistically significant, was observed in WAS T cells plated on fibronectin, in the presence of the lowest dose of anti-CD3 mAb (0.1 $\mu\text{g/ml}$).

Altogether, these data indicate that WASP deficiency does not significantly impairs neither the ability of T cells to adhere to anti-CD3 mAb nor to fibronectin.

Figure 21

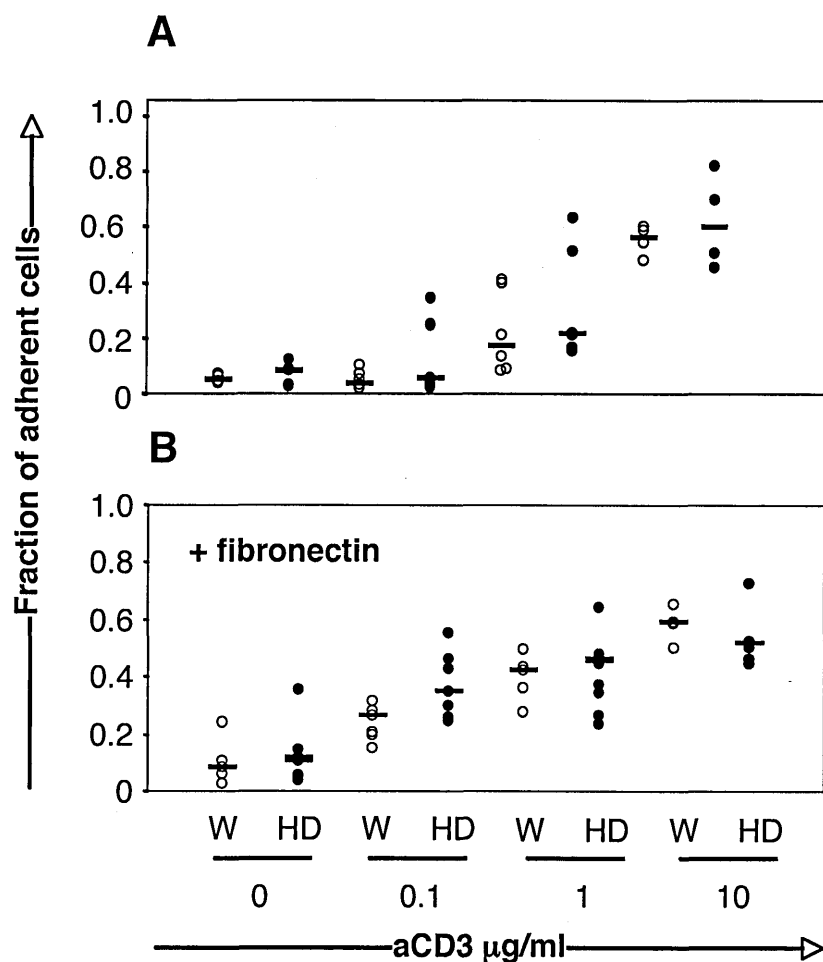


Figure 21. Adhesion of WAS T cell lines to immobilized anti-CD3 or anti-CD3 mAb plus fibronectin. Resting T cell lines from patients W1 and W2 (W) and from three healthy donors (HD) were left to adhere to immobilized anti-CD3 at the indicated doses in the absence (A) or presence (B) of fibronectin, for 30 min. After removal of non-adherent cells, the fraction of adherent cells was calculated. Results correspond to four independent experiments using cells from two WAS patients and from the same healthy donors.

4.3.5 WASP is required for IL-2 and IFN- γ gene transcription by CD4⁺ T cell lines

Defective IL-2 secretion in T cells from WASP knock-out mice were shown to be due to a block in IL-2 mRNA expression (Cianferoni et al., 2005; Morales-Tirado et al., 2004). However, no data were available on the expression of IL-2 mRNA and of mRNA for other cytokines in human WASP-deficient cells. To address this point, and to further analyze the mechanisms responsible for the dichotomy of Th1 versus Th2 cytokine production observed in WAS CD4⁺ T cells, cytokine mRNA levels were analyzed by RNase protection assay (RPA) in CD4⁺ T cell lines from patients W1 and W2 and from two healthy donors (Figure 22A). Quantification in one representative experiment (Figure 22B) shows that upon TCR/CD28-mediated stimulation, levels of both IL-2 and IFN- γ mRNA were lower in WAS CD4⁺ T cells, as compared to control CD4⁺ T cells. This reduction was observed in three independent experiments (W1: IL-2 mRNA=10-52% of the normal values; W2: IL-2 mRNA=22-63% of the normal values). In contrast, IL-4 mRNA was expressed by WAS CD4⁺ T cells at higher levels than those observed in control T cells, while production of IL-10 mRNA was in the normal range (Figure 22B). Independently from the absolute values, CD4⁺ T cells from WAS patients stimulated through TCR/CD28 reproducibly showed higher IL-4 *versus* IFN- γ mRNA ratio than control CD4⁺ T cells, indicating a difference in the balance between these two cytokines. In accordance to what observed with the secreted cytokines, stimulation with TPA plus ionomycin induced comparable expression of the different mRNA transcripts in WAS and control CD4⁺ T cells (Figure 22B).

Therefore, defective IL-2 and IFN- γ production, after TCR/CD28 triggering, in WAS CD4⁺ T cells is determined at the transcriptional level.

Figure 22

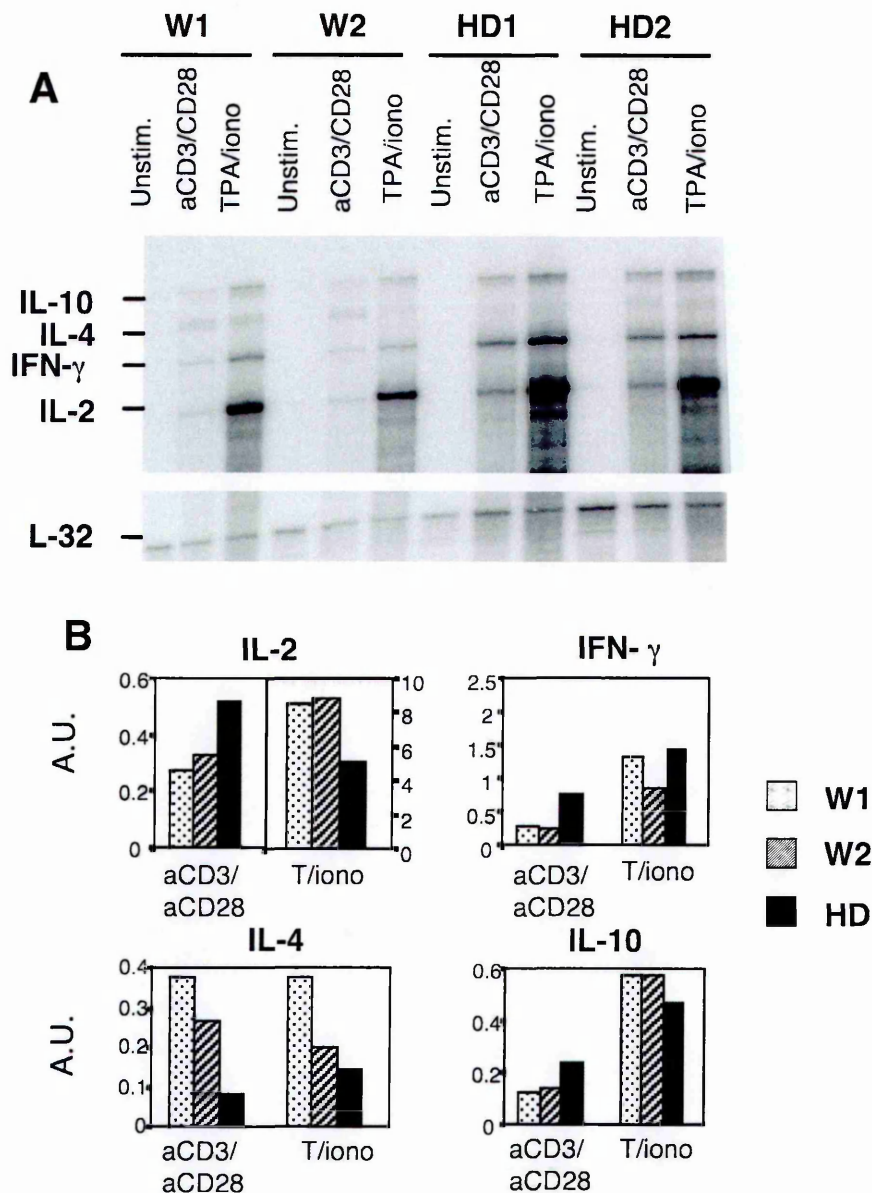


Figure 22 . Analysis of cytokine mRNA expression in WAS CD4⁺ T cell lines. RNase protection assay of the indicated mRNA species extracted from WAS and normal CD4⁺ T cell lines, stimulated with 1 μ g/ml imm. anti-CD3 plus 10 μ g/ml soluble anti-CD28 mAbs or TPA plus ionomycin (A). Results obtained from densitometric analysis were expressed as a ratio of each cytokine value versus the corresponding L32 value. mRNA levels of IL-2, IFN- γ , IL-4 and IL-10 (B) are shown. Background values were subtracted. One representative experiment out of three is shown. Mean value of the two healthy donors is shown for each mRNA species analyzed.(A.U.: arbitrary units).

4.3.6 WASP is required for IL-2 and IFN- γ gene transcription by CD8⁺ T cell lines

To investigate whether a similar mechanism accounted for defective production of IL-2 and IFN- γ in WAS CD8⁺ T cells, RPA was performed on CD8⁺ T cell lines from the same patients (W1 and W2) and healthy controls (Figure 23A). Similarly to what observed for WAS CD4⁺ T cells, following anti-CD3/CD28 mAb stimulation, WAS CD8⁺ T cells expressed lower levels of IL-2 and IFN- γ mRNA, as compared to control CD8⁺ T cells (Figure 23B). The defect was not present when cells were stimulated with TPA plus ionomycin (Figure 23B).

These findings indicate that defective IL-2 and IFN- γ production in WAS CD8⁺ T cells is also dependent on TCR/CD28-mediated stimulation, and is determined at the transcriptional level.

Figure 23

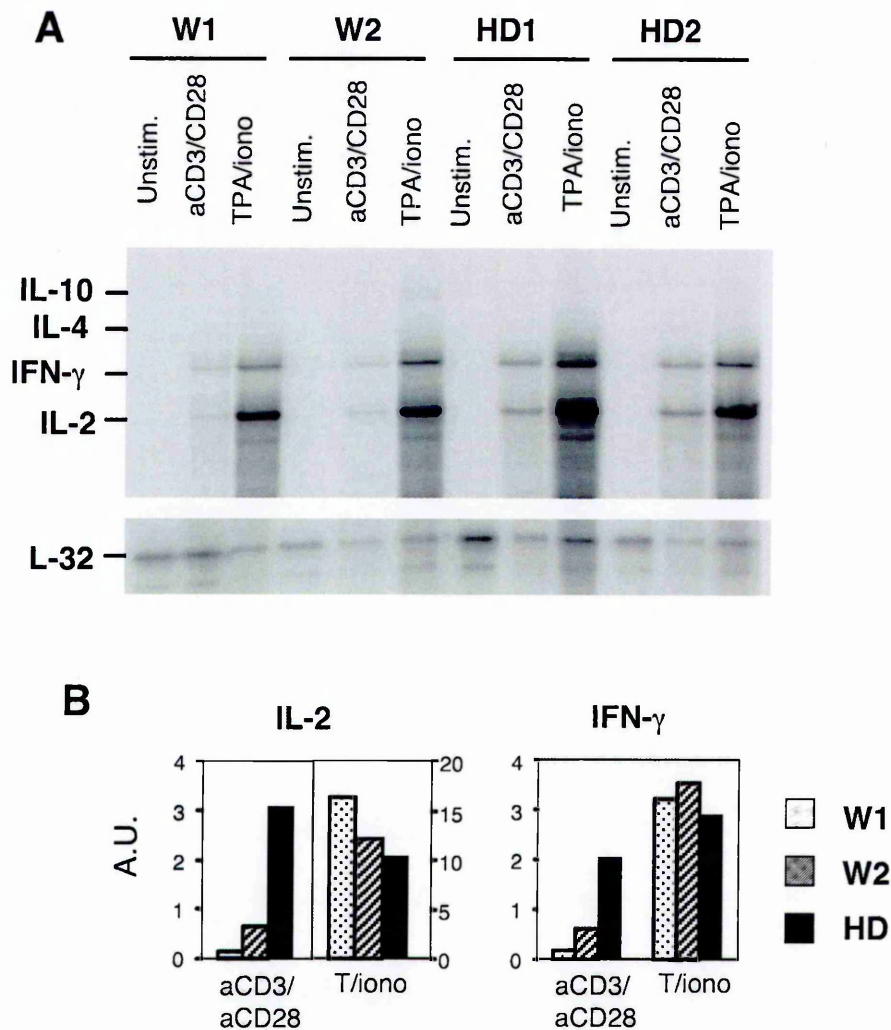


Figure 23. Analysis of cytokine mRNA expression in WAS CD8⁺ T cell lines. RNase protection assay of the indicated mRNA species extracted from WAS and normal CD8⁺ T cell lines, stimulated with 1 μ g/ml imm. anti-CD3 plus 10 μ g/ml soluble anti-CD28 mAbs or TPA plus ionomycin (A). Results obtained from densitometric analysis were expressed as a ratio of each cytokine value versus the corresponding L32 value. mRNA levels of IL-2, IFN- γ (B) are shown. Background values were subtracted. One representative experiment out of three is shown. Mean value of the two healthy donors is shown for each mRNA species analyzed.(A.U.: arbitrary units).

4.3.7 Normal ERK1/2 phosphorylation in CD4⁺ and CD8⁺ T cell lines from WAS patients

To further investigate the consequences of impaired IS formation in WAS T cells at the molecular levels, phosphorylation of the MAPKs ERK1/2 was analyzed in WAS and control CD4⁺ and CD8⁺ T cell lines, upon stimulation with beads coated with anti-CD3 alone (10 µg/ml) or with anti-CD3 (1 µg/ml) plus anti-CD28 mAb (Figure 24). Stimulation of control CD4⁺ T cells with high doses anti-CD3 mAb or anti-CD3 plus anti-CD28 mAb induced ERK1/2 phosphorylation (Figure 24). In WAS T cells, similar levels of phosphorylated ERK1/2 were detected (Figure 24). In WAS and control CD8⁺ T cells, stimulation with high anti-CD3 mAb doses induced comparable levels of phosphorylation (Figure 24). Stimulation of CD8⁺ T cells with a lower anti-CD3 mAb dose plus anti-CD28 mAb induced lower levels of ERK1/2 phosphorylation, which were however comparable between WAS and control T cells. Overall, these results indicate the activation of the ERK1/2 pathway is conserved in WAS T cells.

Since sustained ERK1/2 activation has been shown to be required for IFN-γ production, but not for IL-4 production (Badou et al., 2001), ERK1/2 phosphorylation was analyzed also at late time points of activation in WAS CD4⁺ T cells, in order to verify whether differential IL-4 versus IFN-γ production in these cells could be related to inability to sustain ERK activation. As shown in Figure 25, stimulation with anti-CD3 plus anti-CD28 mAb-coated beads induced phosphorylation of ERK1/2 in both control and WAS CD4⁺ T cells, which was maintained, at comparable levels, up to four hours.

These data show that overall activation of ERK1/2 is preserved in WAS CD4⁺ and CD8⁺ T cells, indicating that the signalling leading to the activation of these molecules, downstream of TCR/CD28, is functional in WAS T cells.

Figure 24

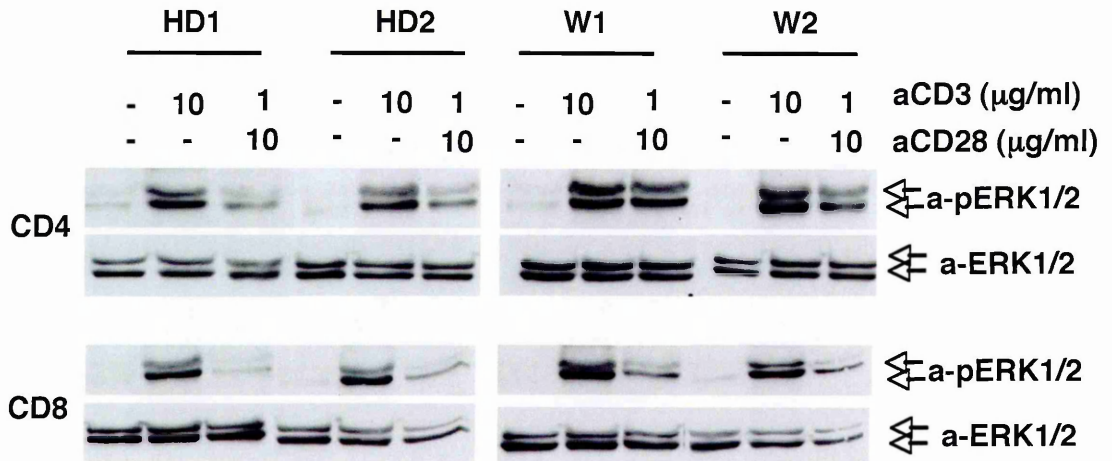


Figure 25

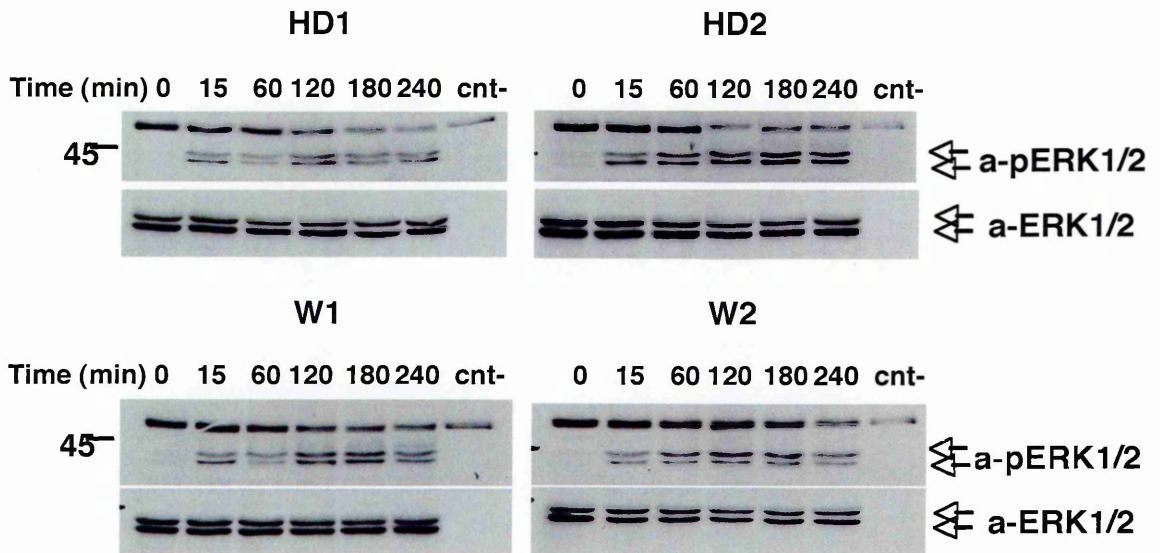


Figure 24. Analysis of ERK1/2 phosphorylation in WAS CD4⁺ and CD8⁺ T cell lines. Serum-starved CD4⁺ and CD8⁺ T cell lines from patients W1 and W2 and from two healthy donors (HD) were stimulated with beads coated with 1 µg/ml anti-CD3 plus 10 µg/ml anti-CD28 mAb for 15 min. Cell lysates were resolved by SDS PAGE and phosphorylation of ERK1/2 MAPK was assessed, after immunoblotting, by sequentially probing with anti-phospho ERK Ab (a-pERK1/2) and anti-panERK Ab (a-ERK1/2). The two bands corresponding to p44 and p42 ERK are indicated by arrows. One representative experiment out of two is shown.

Figure 25. Analysis of sustained ERK1/2 phosphorylation in WAS CD4⁺ T cell lines. Serum-starved CD4⁺ T cell lines from patients W1 and W2 and from two healthy donors (HD) were stimulated with beads coated with 1 µg/ml anti-CD3 plus 10 µg/ml anti-CD28 mAb for the indicated times. Cell lysates were resolved by SDS PAGE and phosphorylation of ERK1/2 MAPK was assessed, as described for figure 24.

4.3.8 Normal JNK1/2 phosphorylation in CD4⁺ T cell lines from WAS patients

Since Cdc42 has been shown to activate JNK1/2 MAPKs (Minden et al., 1995), it is possible that WASP plays a role in transmitting the signals from activated Cdc42 to these kinases. Therefore, the phosphorylation of these MAPKs was also analyzed in cytosolic extracts of WAS and control T cells, stimulated with beads coated with anti-CD3, in the presence of anti-CD28 mAb, or with TPA plus ionomycin (Figure 26). Phosphorylation of both JNK1 and JNK2 was observed, after 30 minutes of stimulation through TCR/CD28 in both WAS and control CD4⁺ T cells. After 120 minutes of stimulation, phosphorylated JNK1/2 were not detectable anymore neither in WAS nor in control CD4⁺ T cells. The pattern of phosphorylation was comparable in WAS and control cells tested (Figure 26). Stimulation with TPA plus ionomycin induced robust phosphorylation of p46 JNK1/2 both in WAS and in control CD4⁺ T cells. Together, these data indicated that, consistently with what observed in T cells from WASP knock-out mice (Zhang et al., 1999), WASP deficiency does not lead to evident alterations in the activation of JNK1/2 MAPKs.

Figure 26

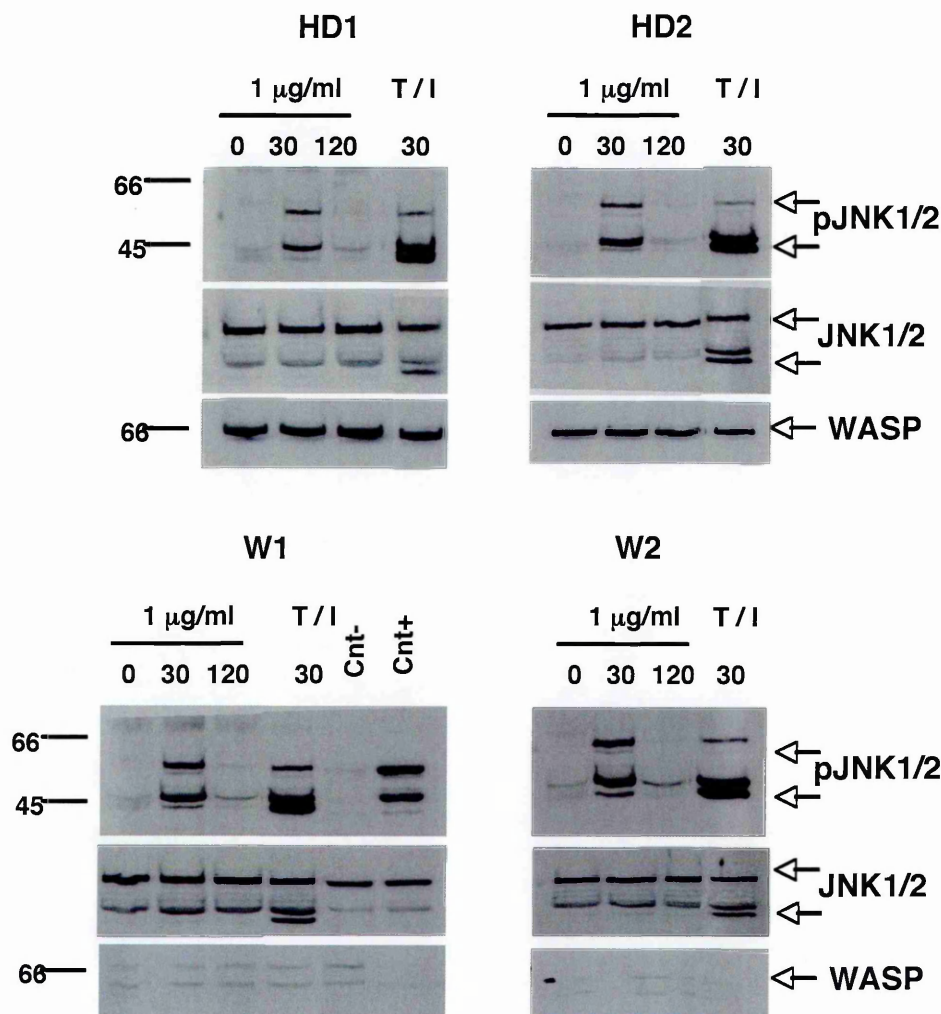


Figure 26. Analysis of JNK1/2 phosphorylation in WAS CD4⁺ T cell lines. Serum-starved CD4⁺ T cell lines from patients W1 and W2 and from two healthy donors (HD) were stimulated with beads coated with 1 µg/ml anti-CD3 plus 10 µg/ml anti-CD28 mAb, or with TPA plus ionomycin, for the indicated times. Cell lysates were resolved by SDS-PAGE and phosphorylation of JNK1/2 MAPK was assessed, after immunoblotting, by sequentially probing with anti-phospho JNK1/2 Ab and anti-panJNK1/2 Abs. The two bands corresponding to p46 and p52 JNK1/2 are indicated by arrows. Positive and negative controls of JNK1/2 phosphorylation are indicated as “Cnt”. The same membranes were then re-probed by anti-WASP Ab.

4.3.9 Normal TCR-triggered Ca^{2+} flux in CD4^+ and in CD8^+ T cell lines from WAS patients

In previous experiments we observed a mild defect in Ca^{2+} flux of bulk WAS T cell lines, stimulated through TCR and CD28 (Dupre et al., 2002). Since it is possible that CD4^+ and CD8^+ have intrinsically different responses in terms of Ca^{2+} flux, the levels of Ca^{2+} were analyzed in resting CD4^+ (Figure 27A) and in CD8^+ (Figure 27B) T cells stimulated with cross-linked anti-CD3 mAb. Analysis of the mean fluorescence intensity (MFI) of the Ca^{2+} -binding dye Fluo3 showed that the levels of Ca^{2+} , analyzed either at the peak or 400 seconds after the stimulation, were normal in both CD4^+ and in CD8^+ T cells. These data differ from those we previously obtained, although it should be noted that in this second set of experiments stimulation was performed by anti-CD3 mAb only. Therefore, it may possible that a defect in Ca^{2+} flux is present in WAS T cells only upon stimulation through TCR and CD28. Further experiments will be required to address this point. However, it should be noted that the effect of WASP deficiency on Ca^{2+} flux in T cells, has been quite controversial, as it will be discussed in the discussion section.

Figure 27

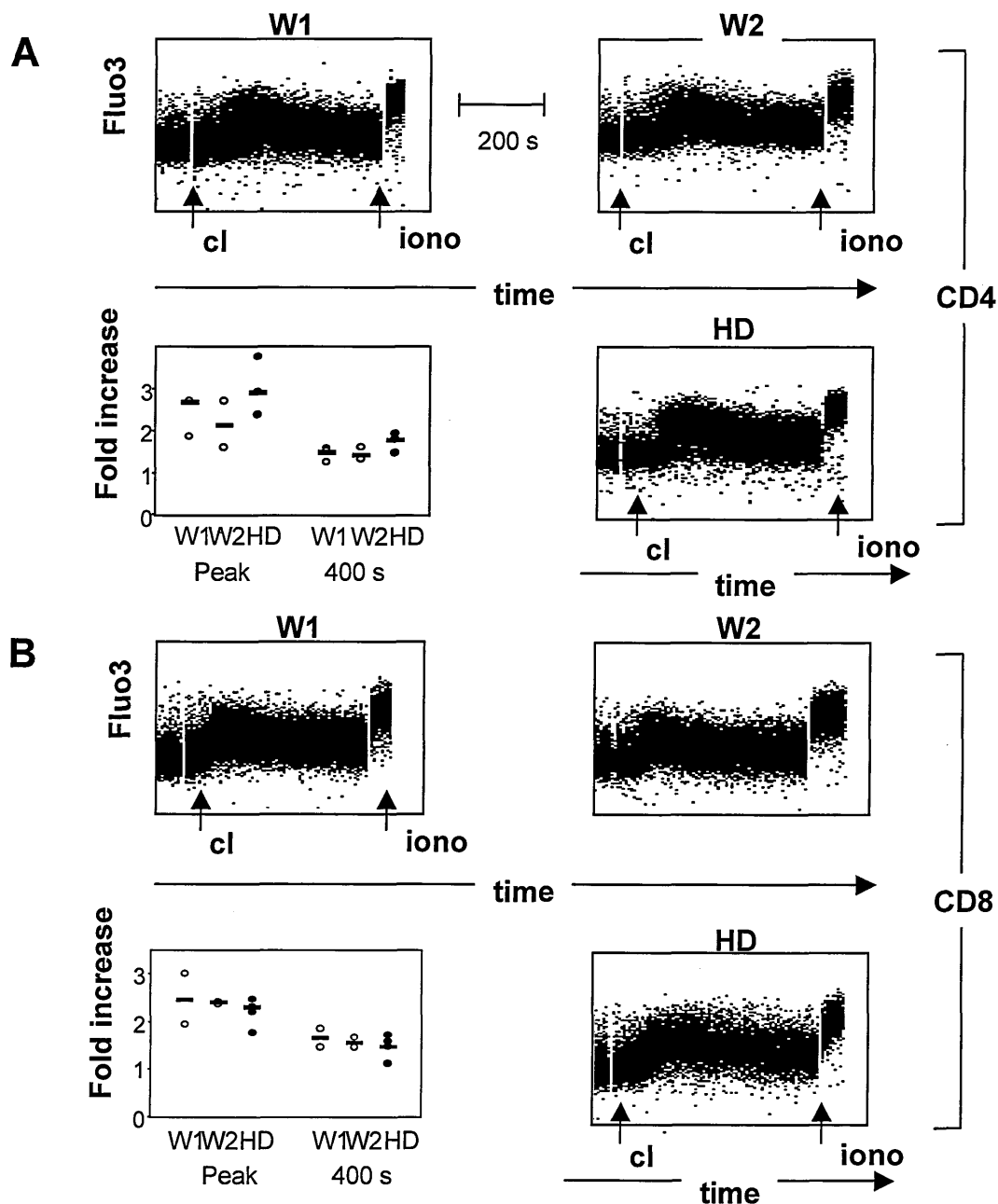


Figure 27. FACS analysis of TCR-elicited Ca^{2+} flux in WAS CD4^+ and CD8^+ T cell lines. Ca^{2+} flux in CD4^+ (A) and CD8^+ (B) T cell lines from patients W1 and W2 and from one (out of two) healthy donor (HD), stimulated with crosslinked anti-CD3 mAb, is shown. Levels of Fluo3 fluorescence were recorded for 400 s ca., then ionomycin was added. MFI calculated at the peak and after 400 s of stimulation is shown for CD4^+ (A) and CD8^+ (B) T cells. Results from two independent experiments are shown. Values corresponding to WAS patients are shown separately, while values corresponding to healthy donors are collectively shown as HD. Cl: crosslinking.

4.3.10 Reduced NFAT nuclear levels in CD4⁺ and in CD8⁺ WAS T cell lines

Since defective NFAT-1 activation and Fos expression have been recently shown to be associated to defective IL-2 gene transcription in T cells from WASP knock-out mice (Cianferoni et al., 2005), we investigated the nuclear levels of NFAT family members (NFAT-1 and NFAT-2) and Fos family members in T cell lines from WAS patients. Recruitment of the dephosphorylated forms of NFAT-1 and NFAT-2 to the nucleus was observed in both WAS and control CD4⁺ T cells, as early as 10 min after stimulation through TCR/CD28 and persisted for at least 120 min (Figure 28A). However, in WAS CD4⁺ T cells, the levels of nuclear NFAT-1 were lower than in control CD4⁺ T cells at the 10 min time point (Figure 28B). This difference was observed in three out of four independent experiments. At later time points, comparable levels of nuclear NFAT-1 were detected in WAS and control CD4⁺ T cells. Nuclear recruitment of NFAT-2 was observed after a 10-min stimulation and persisted for at least 120 min, and it was comparable in WAS and control CD4⁺ T cells (Figure 28A and 28B). WAS and control CD4⁺ T cells showed a similar profile of expression of Fos family members, which were induced at 29 min and further increased 120 min after stimulation (Figure 28A and 28B). In WAS CD8⁺ T cells, a significant reduction in the nuclear levels of both NFAT-1 and NFAT-2 were observed 10 min after TCR/CD28 triggering (Figure 29A and 29B; NFAT-1 $p < 0.05$; NFAT-2 $p < 0.01$). Although in WAS CD8⁺ T cells, decreased levels of NFAT-1 and NFAT-2 were also found at later time points (Figure 29A and 29B), this reduction was not significant. Fos family members were expressed at similar levels in WAS and control CD8⁺ T cells 120 min after TCR/CD28 triggering (Figure 29A and 29B). These data indicate that the absence of WASP in human CD4⁺ and CD8⁺ T cells, stimulated through TCR/CD28, leads to a

reduction in the early nuclear recruitment of NFAT proteins, while the expression of Fos proteins appears normal.

Figure 28

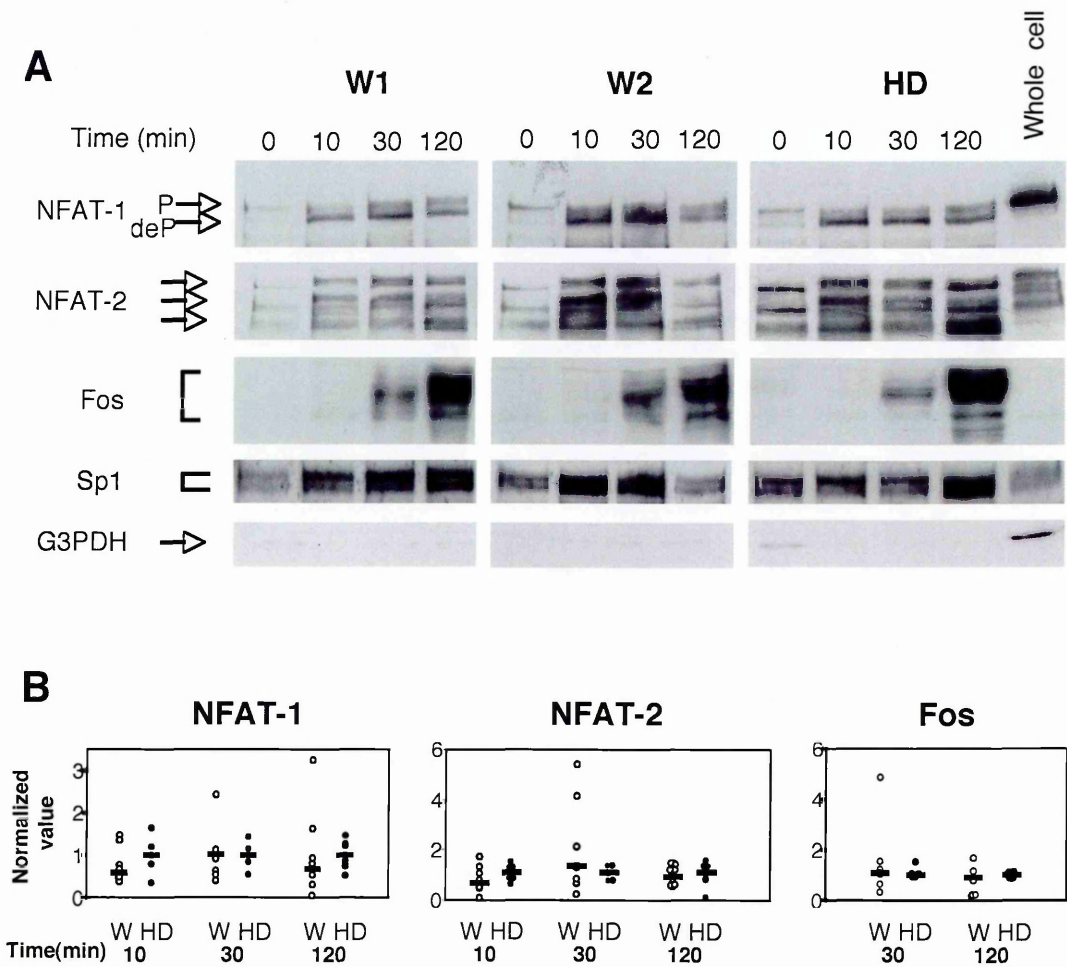


Figure 28. Analysis of expression levels of NFAT-1, NFAT-2 and Fos in the nuclei of WAS and control CD4⁺ T cell lines stimulated via TCR and CD28. Levels of NFAT-1, NFAT-2 and of Fos family members in the nuclei of CD4⁺ T cell lines from WAS patients and healthy donor (A) stimulated by anti-CD3 plus anti-CD28 mAbs-coated beads for the indicated times are shown. Membranes were blotted with anti-NFAT-1, anti-NFAT-2 and anti Fos Abs, then re-probed with anti-Sp1 and anti-G3PDH Abs, to check nuclear and cytosolic separation. One (out of two) healthy donor is shown for each experiment. As control, whole cell lysate from control resting T cells is shown (A). Densitometric analysis of three to four independent experiments is shown (B).

Figure 29

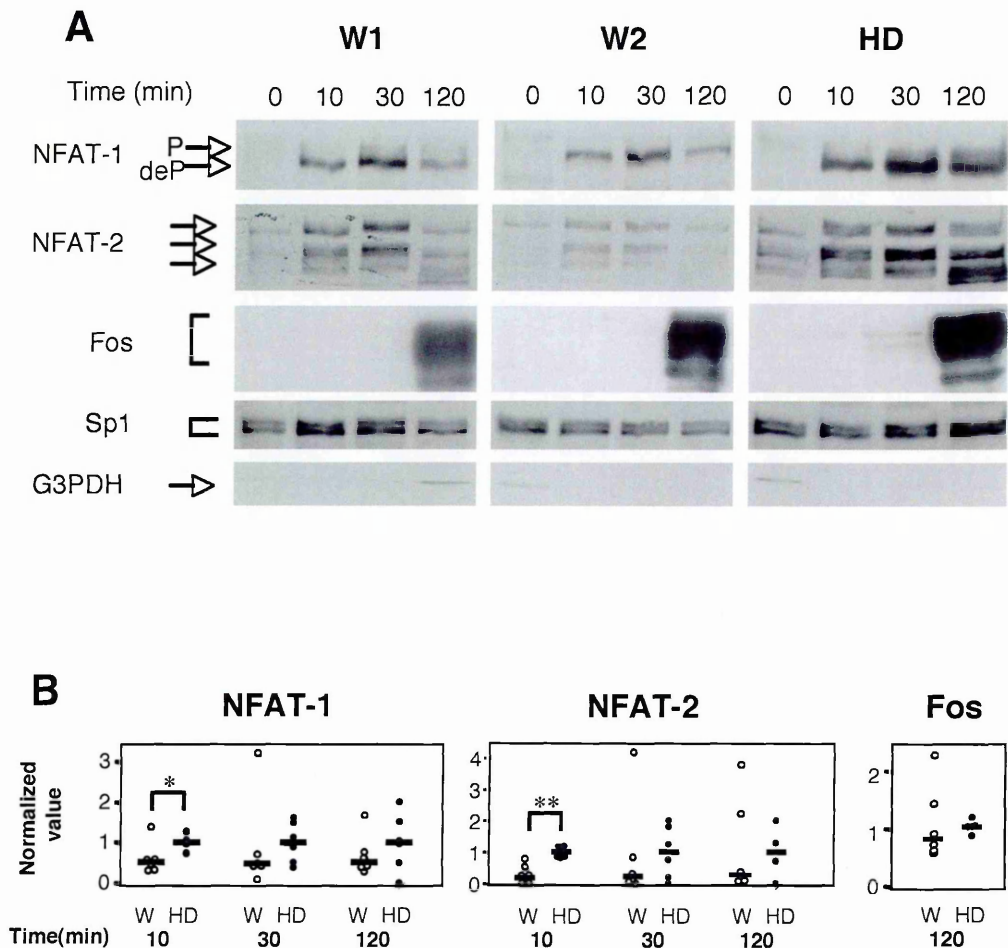


Figure 29. Analysis of expression levels of NFAT-1, NFAT-2 and Fos in the nuclei of WAS and control CD8⁺ T cells stimulated via TCR and CD28. Levels of NFAT-1, NFAT-2 and of Fos family members in the nuclei of CD8⁺ T cell lines from WAS patients and healthy donor (A) stimulated by anti-CD3 plus anti-CD28 mAbs for the indicated times are shown. Membranes were blotted with anti-NFAT-1, anti-NFAT-2 and anti Fos Abs, then re-probed with anti-Sp1 and anti-G3PDH Abs, to check nuclear and cytosolic separation. One (out of two) healthy donor is shown for each experiment. Densitometric analysis of three independent experiments is shown (B).

4.3.11 Prolonged I κ B α phosphorylation in WAS CD4⁺ T cell lines upon

TCR/CD28 triggering

We then analyzed the activation of another family of transcription factor, NF κ B, which is involved in the transcription of IL-2 and other cytokine genes (see section 1.5.3.7.5). In resting cells NF κ B proteins are retained in the cytoplasm through binding to I κ B proteins. Upon receptor triggering, I κ B proteins are rapidly phosphorylated and therefore targeted for ubiquitination and proteasome-mediated degradation. I κ B degradation allows nuclear translocation of NF κ B dimers. We then analyzed the phosphorylation of I κ B α induced by stimulation of WAS and control CD4⁺ T cells with beads coated with anti-CD3 plus anti-CD28 mAb. As shown in figure 30A, after 30 minutes of stimulation, I κ B α is phosphorylated in both control and WAS CD4⁺ T cells, but at slightly higher levels in the latter (Figure 30B). After 120 minutes of stimulation, levels of phosphorylated I κ B α were higher in WAS with respect to control CD4⁺ T cells (Figure 30A and 30B).

Higher phosphorylation of I κ B α could be the result of defective I κ B α degradation, or more in general, to altered turnover of the protein. Although in WASP knock-out murine T cells normal NF κ B activity has been reported (Cianferoni et al., 2005), our results suggest that WASP deficiency could alter NF κ B activity. Further experiments will be necessary to address this point.

Figure 30

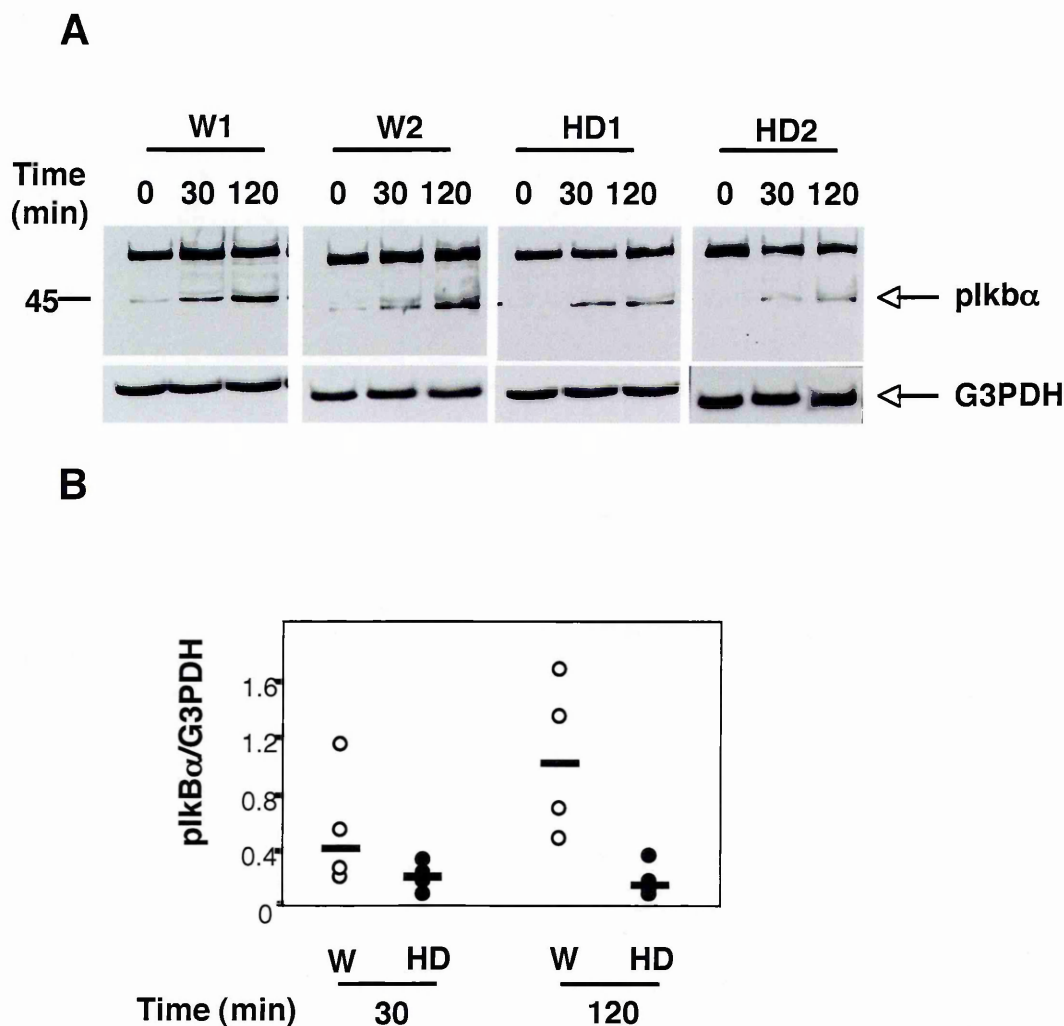


Figure 30. Analysis of phosphorylation of I κ B α in WAS and control CD4⁺ T cell lines stimulated via TCR and CD28. Resting T cell lines from patients W1 and W2 and from two healthy donors (HD) were stimulated with beads coated with 1 μ g/ml anti-CD3 plus 10 μ g/ml anti-CD28 mAb for the indicated times and cytosolic extracts were resolved by SDS-PAGE. After blotting, phosphorylation of I κ B α was detected by specific Ab (A). Membranes were re-probed with anti-G3PDH Ab to normalize for the amount of proteins loaded (A). Densitometric analysis of two independent experiments is shown (B). Values from WAS patients (W) and from healthy donors (HD) are collectively shown.

4.3.12 Reduced T-bet mRNA induction in WAS CD4⁺ T cell lines stimulated via TCR and CD28

We next analyzed the levels of T-bet and GATA-3 mRNA in WAS CD4⁺ T cell lines, as these transcription factors are involved in the transcription of IFN- γ and IL-4 genes, respectively (Szabo et al., 2002; Zheng and Flavell, 1997). Levels of both T-bet and GATA-3 mRNA were comparable in resting CD4⁺ T cells from W1 and W2 patients and healthy controls (Figure 31A and 31B). Upon stimulation with 1 μ g/ml anti-CD3 plus 10 μ g/ml anti-CD28 mAb, T-bet was induced in both control and WAS CD4⁺ T cells, but at a lower levels in the latter (Figure 31A, $p < 0.05$ for patient W2 compared to healthy donors). At suboptimal dose of anti-CD3 (0.1 μ g/ml) plus anti-CD28 mAb, T-bet mRNA expression was significantly higher in control cells than in WAS CD4⁺ T cells (Figure 31A, $p < 0.05$ for both W1 and W2 patients). Stimulation with TPA plus ionomycin induced comparable T-bet expression in WAS and control CD4⁺ T cells (data not shown). At high doses of anti-CD3 mAb (1 μ g/ml), GATA-3 mRNA was expressed by WAS and control cells at comparable levels (Figure 31B). At the lower anti-CD3 mAb dose (0.1 μ g/ml), GATA-3 mRNA was not induced in WAS CD4⁺ T cells whereas only a fraction of control T cells expressed it (Figure 31B). These data show that CD4⁺ T cells from WAS patients have a reduced ability to induce T-bet mRNA upon TCR/CD28-mediated stimulation.

Figure 31

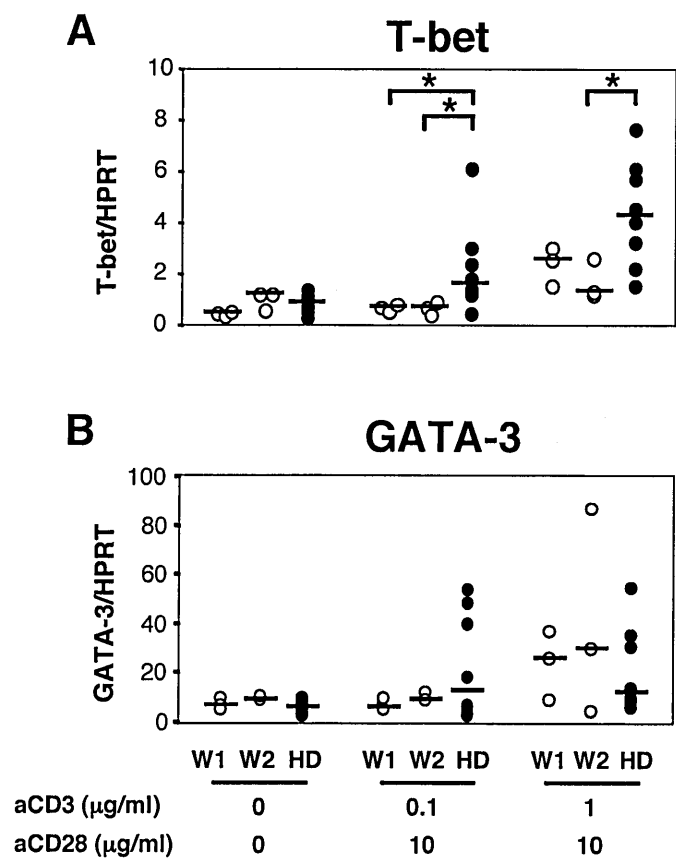


Figure 31. Analysis of T-Bet and GATA-3 relative mRNA levels in WAS CD4⁺ T cell lines upon TCR/CD28-mediated stimulation. Measurement of T-bet (A) and of GATA-3 (B) gene expression before and after TCR/CD28-mediated stimulation of CD4⁺ T cells from patients W1 and W2 and two healthy controls. Total RNA was prepared from either resting or stimulated cells and gene expression was measured by semi-quantitative RT-PCR. Values were normalized on expression of the housekeeping gene HPRT and correspond to duplicate wells. Results derived from three independent experiments are shown. Values of three healthy donors are collectively shown as HD. Statistical significance is represented as follows: * p<0.05; ** p<0.01

All together, these studies on the cellular and molecular mechanisms involved in WAS T cells unresponsiveness to TCR/CD28-mediated stimulation, indicate a role of WASP in the modulation of GM1 levels during T cell activation. In addition, WASP deficiency affects the activation of different transcription factors, which is likely to contribute to the impairment in the transcriptional activation of Th1 cytokine genes.

4.4 Cellular mechanisms of WAS-associated autoimmunity: a role for natural T regulatory (nTreg) cells?

4.4.1 CD4⁺/CD25⁺/FoxP3⁺ cells in the peripheral blood of WAS patients

Studies performed in our laboratory revealed a defect in the function of CD4⁺/CD25⁺/FoxP3⁺ nTreg cells isolated from the spleen of WASP knock-out mice (Marangoni F., *Personal communication*). WAS is often associated to autoimmune manifestations and therefore we hypothesized that alterations of nTreg cells could be present in WAS patients (see section 1.7). First, the presence of nTreg in the blood of different WAS patients was investigated, based on the presence of cells expressing of CD25 and of the forkhead transcription factor FoxP3. Percentages of CD4⁺/CD25^{bright} cells in the blood of WAS patients were comparable to percentages observed in age-matched healthy donors (not shown). The percentages of CD4⁺/FoxP3⁺ cells in PBMC of WAS patients were higher among PBMC of WAS patients than in PBMC of healthy controls (Figure 32A and 32B). The percentages of FoxP3⁺/CD25⁺ cells were also slightly higher in the blood of WAS patients than in healthy controls, although this difference was not significant (Figure 32B). However, it should be considered that some of these patients are lymphopenic (W1 and W14) meaning that the absolute numbers of nTreg cells among total PBMCs can be lower than the normal. The analysis is currently being extended to other WAS patients. It would be also important to evaluate the proportion of nTreg cells in the secondary lymphoid organs of WAS patients.

Figure 32

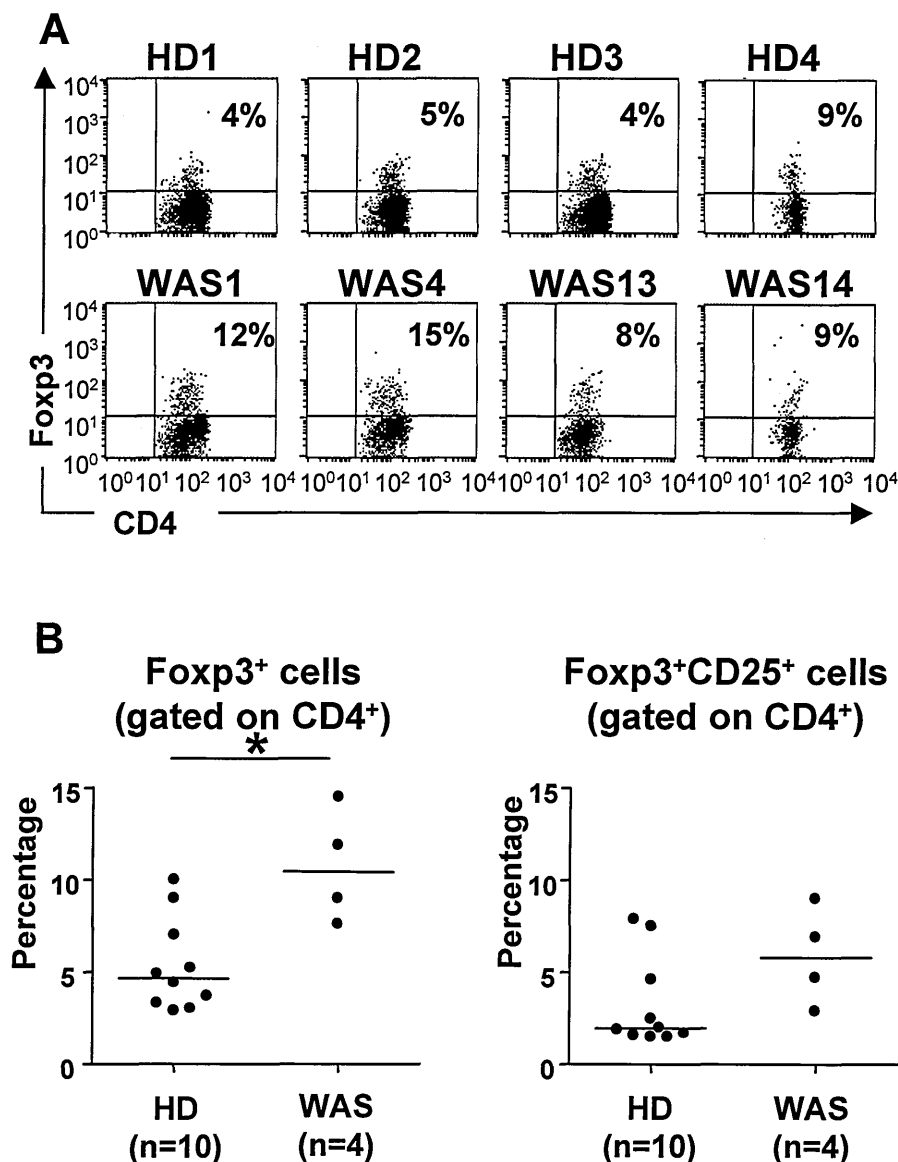


Figure 32. Analysis of WAS nTreg phenotype: CD25 and FoxP3 expression. PBMC from WAS patients and from age-matched healthy donors were stained with anti-CD4 FITC and anti-FoxP3 PE Abs. Analysis of Foxp3 expression in CD4⁺ population is shown (A). Analysis of the percentages of FoxP3⁺ and FoxP3⁺/CD25⁺ cells in four WAS patients and 10 healthy donors is shown (B). Statistical significance is represented as follows: * $p < 0.05$.

4.4.2 Defective suppressor activity of nTreg cells from WAS patients

We next investigated the ability of freshly isolated nTreg from WAS patients and age-matched healthy donors to suppress proliferation by allogenic CD4⁺/CD25⁻ effector T cells, isolated from healthy donors. As shown in Figure 33A, nTreg cells isolated from healthy donors were able to suppress proliferation of allogenic effector T cells (range of suppression: 39-85%) at higher levels than the nTreg cells from the two WAS patients analyzed (range of suppression: 0-10%). Stimulation of nTreg alone from either WAS patients or healthy donors did not elicit significant proliferation, confirming that these cells are anergic *in vitro*, as previously reported (Levings et al., 2002). The mechanisms underlying defective suppression by nTreg cells from WAS patients remain to be clarified. However, data obtained in our group with nTreg cells from WASP knock-out mice indicate that WASP absence leads to defective activation of nTreg cells upon stimulation with anti-CD3 mAb plus anti-CD28 (Marangoni F., *Personal communication*). The ability of human WAS nTreg cells to suppress IFN- γ secretion by allogenic effector T cells was also reduced *in vitro*, although to a lesser extent compared to the ability to suppress proliferation, tested in the same assays (Figure 33B). It is possible that the ability to suppress proliferation or cytokine secretion by nTreg cells is controlled by different mechanisms, or even it requires different levels of activation of nTreg cells. Future studies in additional WAS patients are required to confirm and clarify this point.

Figure 33

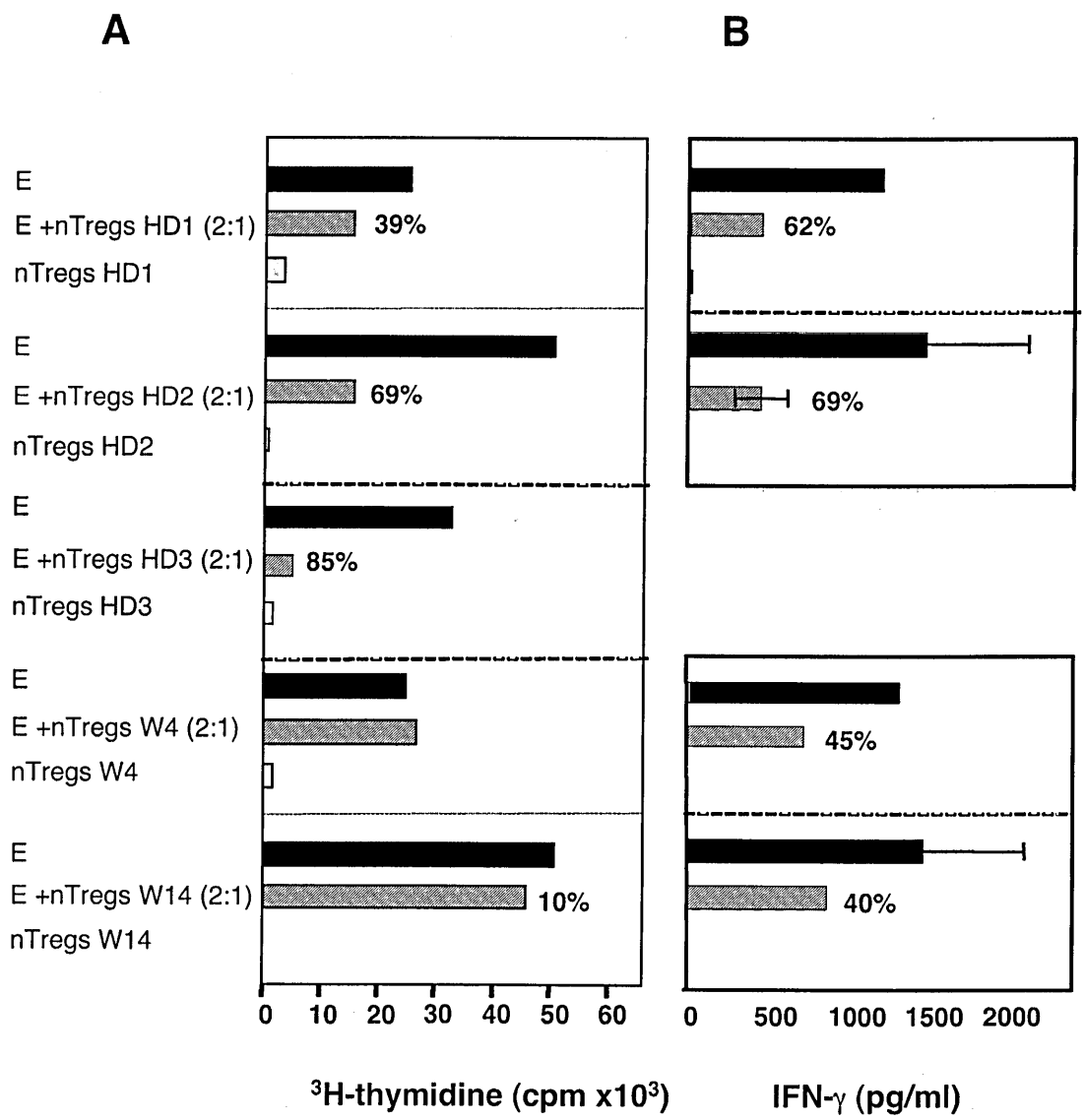


Figure 33. Analysis of the suppressive capacity of WAS nTreg cells directed toward allogenic effector cells. CD4⁺/CD25⁺ nTreg cells were isolated from the peripheral blood of WAS patients and age-matched healthy donors. The ability of nTreg cells to suppress the proliferation of allogenic CD4⁺/CD25⁺ effector cells, stimulated with either anti-CD3 mAb plus APC (W4, HD1 and HD3) or with anti-CD3/28 mAb-coated beads (W14 and HD2) was evaluated after 72 hs of stimulation. In parallel, levels of IFN- γ in the supernatants of the cultures was measured after 72 hs of stimulation. Percentages of suppression are indicated.

To further investigate whether WAS-associated autoimmune manifestations are due to the inability of WAS nTreg cells to suppress the response of autologous effector T cells, the suppressive capacity of W4 nTreg cells was evaluated using as effector T cells autologous CD4⁺/CD25⁻ cells. Figure 34 shows that the ability of W4 nTreg cells to suppress both proliferation and IFN- γ secretion by autologous effector cells was lower than the one of control nTreg cells.

Figure 34

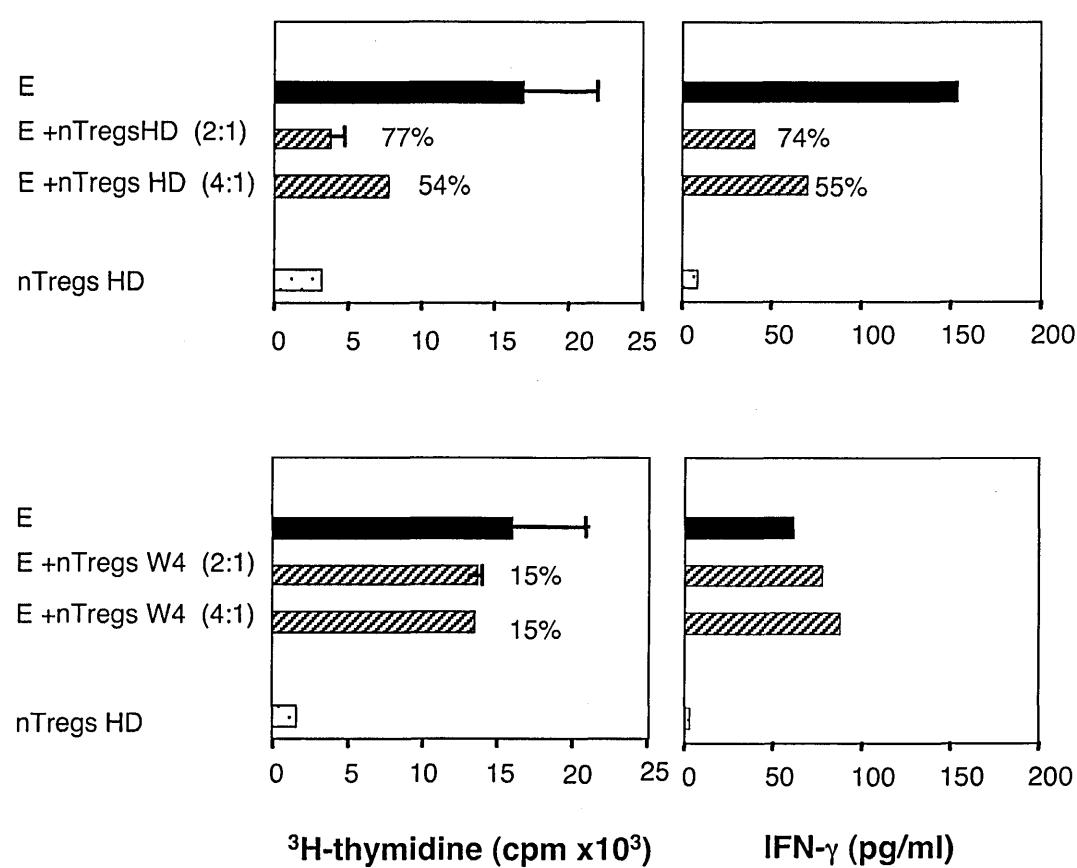


Figure 34. Analysis of the ability of WAS nTreg cells to suppress the proliferation of autologous effector cells. CD4⁺/CD25⁺ nTreg cells were isolated from the peripheral blood of patients W4 and from one age-matched healthy donor. The ability of nTreg cells to suppress the proliferation of autologous CD4⁺/CD25⁻ effector cells, stimulated with anti-CD3 mAb plus APC was evaluated after three day-culture. In parallel the ability to suppress IFN- γ secretion was evaluated by collecting the supernatants of the cultures after 72 hs of stimulation.

These data support the possibility that altered regulatory T cell function contributes to the increased susceptibility to autoimmune manifestations in WAS patients and suggest that defective suppressive ability of WAS nTreg cells maybe due to defective activation.

5 DISCUSSION

The results obtained during the course of this PhD project represent new findings, which contribute to the understanding of the role played by WASP in T cell activation, and to gain insight into the cellular and molecular basis of T cell dysfunction in patients affected by WAS. This study indicates that WASP plays a crucial role in the responsiveness of T cells to TCR/CD28 triggering, i) by setting the threshold for T cell activation/proliferation and ii) by influencing the transcriptional activation of cytokine genes. In addition, these results clearly define the effect of WASP deficiency in CD4⁺ Th cells, CD4⁺/CD25⁺ nTr cells and CD8⁺ CTL cells. Here the results obtained will be discussed in view of the existing data and in view of possible novel treatment approaches for WAS patients such as gene therapy.

5.1 The role of WASP in T cell activation mediated by the TCR and CD28

5.1.1 Role of WASP in regulating the proliferative response after anti-CD3/CD28 mAb-mediated activation

Previous studies showed that WASP-deficient T cells, including human T cell lines and T cells isolated from secondary lymphoid organs of WASP knock-out mice, display a defective proliferation to anti-CD3 mAb-mediated stimulation (Molina et al., 1993; Snapper et al., 1998; Zhang et al., 1999). We established T cell lines from WAS and XLT patients, with a broad spectrum of disease severity. For the first time, TCR-mediated activation of WAS CD4⁺ and CD8⁺ T cells was individually studied, using a large scale of anti-CD3 mAb doses, combined or not with anti-CD28 mAb. Our study confirmed that WASP-deficient T cells have a specific defect in response to TCR triggering induced by immobilized anti-CD3 mAb (Figure 2A). Our results also indicated clearly that T cell lines established from a patient with a mild phenotype (XLT) and displaying residual WASP expression showed very limited defect of

proliferation in response to TCR triggering. Importantly, the proliferation defect of WASP-deficient T cells is attributable to both CD4⁺ and CD8⁺ T cells, indicating that these two cell subsets require WASP for optimal activation and proliferation (Figure 2C, 2D, 3C and 3D). Interestingly, a milder proliferation defect was observed in WAS T cell lines upon stimulation with high dose of anti-CD3 mAb in the presence (or absence) of anti-CD28 mAb. These findings, together with the evidence that the defect in proliferation of WAS T cells is more evident at sub-optimal anti-CD3 mAb doses, indicate that WASP lowers the threshold for T cell activation, thus suggesting that WASP is enhancing or sustaining the signals delivered through the TCR. One explanation would be that WASP, through its regulation of actin cytoskeleton, is involved in the re-localization of TCR and associated signalling molecules, which takes place during T cell activation. Our data also indicate that the defective proliferative response to anti-CD3 mAbs of WAS T cells is not associated to a defective TCR downregulation (Figure 2B), or to a defective ability to adhere to immobilized anti-CD3 mAbs (Figure 2I)

Although the addition of anti-CD28 mAb to anti-CD3 mAb stimulation enhanced the proliferative response of WAS T cells (Figure 2D), costimulation was not able to restore normal proliferation in WAS T cells. In our experiments it is difficult to conclude that WAS T cells respond normally to CD28 costimulation, since all the experiments were performed with a single (high) dose of anti-CD28 mAb. However, results obtained in WASP knock-out mice showed that WASP-deficient naïve CD4⁺ T cells required approximately 10 fold higher anti-CD28 mAb concentrations than control cells, in order to respond to proliferate in response to TCR triggering (Morales-Tirado et al., 2004). CD28 costimulation amplifies TCR signalling and therefore it is able to lower the numbers of triggered TCRs, which are necessary for productive T cell activation, making CD28-mediated costimulation particularly required in conditions of

suboptimal TCR engagement (Viola and Lanzavecchia, 1996). Whether costimulation via CD28 is also able to cooperate *in trans* with TCR signalling, via the activation of independently regulated signalling pathways, is still a matter of debate (Michel and Acuto, 2002; Rudd and Raab, 2003). However, Vav-1 is considered a crucial molecule involved in an early connection between TCR and CD28 pathways, which is necessary for signal amplification. This pathway is linked to the remodelling of actin cytoskeleton through the activation of Rac1 and Cdc42. Therefore, WASP could be implied in this pathway through the formation of a Vav-1/SLP76/Nck complex, and therefore being a key molecule involved in TCR/CD28 signal integration. TCR triggering was also shown to induce phosphorylation of WASP on the tyrosine residue 291 (Badour et al., 2004a) and mutation of this residue strongly impaired TCR-mediated NFAT activation. Evidences for a possible role of WASP in transmitting CD28-evoked signals comes from the recent finding that WASP phosphorylation induced upon TCR triggering is strongly enhanced upon CD28 co-engagement (Kim and White, 2006). In addition, WASP has been proposed to regulate CD28 endocytosis in murine T cells (Badour et al., 2004b). Therefore, it would worth to analyze the pathway of CD28 endocytosis in T cells from WAS patients.

Our study helped to clarify the role of IL-2 deficiency in the (partial) inability of WAS T cells to proliferate in response to anti-CD3/CD28 mAb. T cell proliferation is controlled by both TCR/CD28 and IL-2R-mediated signals. Exit from the G1 phase of the cell cycle and entering into the S phase requires IL-2 generated signals (Nourse et al., 1994). Indeed, triggering of the IL-2 receptor induces key events of G1-to-S phase transition, including up-regulation of cyclin D, down-regulation of p27^{Kip}, and hyperphosphorylation of Rb, mediated by PI3K (Brennan et al., 1997). In WAS T cells, defective proliferation correlates with reduced IL-2 production (Figure 8,9,11,12) both in CD4⁺ and CD8⁺ T cells from WAS patients. WAS T cells were able to proliferate

normally to exogenous IL-2, and the addition of IL-2 to sub-optimal doses of anti-CD3 mAb had a synergistic effect on the proliferation of CD8⁺ T cells (Figure 7). This synergistic effect was less clear in WAS CD4⁺ T cells. Thus, WASP deficiency seems not to affect IL-2R signalling and the inability to secrete sufficient amounts of IL-2 is likely to be the main reason for the poor proliferation of WAS T cells. However, it was shown that TCR and CD28-generated signals can mediate G1-to-S phase transition and T cell proliferation independently of IL-2 (Appleman et al., 2000; Boonen et al., 1999). Therefore, it is possible that, in our system, proliferation in response to anti-CD3/CD28 mAb is elicited by both IL-2-dependent and independent mechanisms. Therefore, WASP could be required not only for IL-2 production but also for IL-2 independent mitogenic signals. Therefore, it would be interesting to analyze down-regulation of p27^{Kip} in T cells from WAS patients, stimulated either through TCR/CD28 or IL-2R, in order to further clarify the molecular mechanisms of WASP-mediated regulation of T cell proliferation.

5.1.2 Restoration of physiological levels of WASP expression in WAS T cells by LV gene transfer leads to normal TCR/CD28-induced proliferation

Transfer of WASP-encoding cDNA into T cell lines from WAS patients was performed to verify whether WASP deficiency is directly responsible for the defective proliferation of WAS T cells, and to evaluate the efficacy of RV versus LV gene transfer in view of a future gene therapy approach for WAS patients.

Expression of human WASP into T cell lines derived from WAS patients mediated by either RV and LV gene transfer led to different degrees of correction of the proliferative response after TCR/CD28 stimulation (Figure 4C and 5C). Since correction of proliferation is probably the consequence of restoration of IL-2 production, it is possible that in a mixture of WASP⁺ (transduced) and WASP⁻ (untransduced) cells, the former help the latter to proliferate, by providing a source of

IL-2 production. However, it seems that expression of physiological level of WASP is required to achieve full correction of anti-CD3 mAb-driven proliferation. In accordance with this data, intermediate levels of WASP expression in CD4⁺ and CD8⁺ T cells from the XLT patient (X6) were able to sustain intermediate levels of proliferation, in response to TCR/CD28 triggering (Figure 3C and 3D). We observed that the reduced proliferation of WAS CD4⁺ and CD8⁺ T cells was due to a reduced proportion of cells, which underwent cell division (not shown). This analysis (based on CFSE dilution) could be extended to the cells of XLT patients expressing residual point-mutated WASP, to see whether the expression of a point-mutated WASP correlates with the ability to proliferate. This kind of analysis could be also useful to evaluate more precisely the degree of functional correction of WASP-transduced WAS T cells.

Our results strongly argue in favour of LV as the tools of choice to mediate WAS gene transfer into T cells for a gene therapy purpose. Indeed, LV allowed efficient transduction of T cell lines and freshly isolated PBMC from WAS patients (Figure 6) and long-term gene expression. Importantly, correction of proliferation was achieved by using either the PGK promoter or the autologous WAS promoter (Figure 5C). The latter was able to drive physiological levels of WASP expression with a relatively low numbers of integrants per cell (1 to 3: not shown). This is particularly important in the context of the safety of LV *versus* RV regarding the risk of insertional mutagenesis. Bases on these, and on other results obtained from experiments using an *ex vivo* gene therapy approach in the WASP knock-out mouse (Dupre et al., 2006), the wasp-WASP LV has been chosen for the development of a future gene therapy clinical trial in WAS. In accordance with previous data (Cavalieri et al., 2003), LV were also able to transduce PBMC from WAS patient in the presence of cytokines but in the absence of TCR engagement, thus substantially preserving the naïve phenotype (Figure 6). This feature of LV would be particularly important if gene therapy using peripheral blood

lymphocytes (PBL) is to be envisaged in some of these patients. However, the high frequency of autoimmune manifestations in WAS patients (Dupuis-Girod et al., 2003) should be taken into consideration, as it could reflect the presence of abnormal circulating auto-reactive T cells, possibly due to inefficient negative selection. Therefore, re-introduction of the WAS gene into mature T cells could be deleterious. For this (and for other reasons) the ideal target of gene therapy for WAS will be HSC, in order to allow the development of a balanced immune system and the establishment of tolerance towards the newly expressed protein. However, untransformed T cell lines from WAS patients revealed to be a powerful experimental *in vitro* system for the evaluation of the efficacy (and safety) of different vectors, providing key information for the selection of the vector to be developed for clinical use and helping to define read-outs for the pre-clinical study performed in the laboratory (Dupre et al., 2006).

5.2 Role of WASP in regulating cytokine production in CD4⁺ and CD8⁺ T cells

WASP deficiency in T cells has been linked to defective production of different cytokines in response to TCR/CD28 triggering. Defective IL-2 secretion has been classically considered a hallmark of WASP-deficient T cells. We showed that both WAS CD4⁺ and CD8⁺ T cells accounted for the defective IL-2 production observed in the supernatants of bulk T cell lines after TCR/CD28 triggering (Figure 8, 9, 11 and 12). Impaired IL-2 production was also observed in WAS CD8⁺ T cells stimulated through TCR only (not shown), in accordance with previous data (Strom et al., 2003a). In CD4⁺ T cells from both control and WAS cells, stimulation through TCR alone elicited only minimal IL-2 production (not shown), since this cell subset is highly dependent on CD28-mediated costimulation. In our study, analysis of the pattern of cytokine production by WAS T cells also revealed a strong impairment in the secretion of Th1 cytokines (IFN- γ and TNF- α) both in CD4⁺ and CD8⁺ T cells, after TCR/CD28

stimulation. For IFN- γ we demonstrated that the low levels in the supernatants correspond to reduced protein synthesis (Figure 9 and 12). In accordance with our data, analysis of immune response to Influenza virus in the WASP knock-out mouse model showed that despite normal viral clearance during primary Influenza inoculation occurred, the secondary immune response was defective (Andreansky et al., 2005). In particular, T cells isolated from the spleen of WASP knock-out mice after secondary infection with Influenza virus showed defective production of both IFN- γ and TNF- α .

Previous studies in CD4⁺ T cells from WASP knock-out mice showed that reduced levels of IFN- γ in the supernatants of TCR/CD28-stimulated WAS cells was due to defective secretion rather than to defective synthesis (Morales-Tirado et al., 2004). This was attributed to lack of polarization of IFN- γ and Golgi apparatus, which suggested that altered intracellular localization of this cytokine was responsible for its defective secretion. These data substantially differ from ours, as in our experiments IFN- γ reduction in human WAS T cells is present both at the intra-cellular and extra-cellular level, thus arguing against a defect of secretion. Moreover, treatment of WAS T cells with phorbol ester (TPA) and Ca²⁺ ionophore (ionomycin) was able to trigger high levels of IFN- γ production (Figure 9 and 12) and secretion (not shown) in WAS T cells, indicating that IFN- γ production could be restored, likely bypassing defective signalling via the TCR. However, we cannot rule out the possibility that, in WAS T cells stimulated with TPA plus ionomycin, vesicles containing IFN- γ are secreted without the correct polarization of the secretory apparatus. Recent data indicate that secretion of IL-2 and IFN- γ follows a different pathway from the one followed by IL-4 and TNF- α . Indeed, the vesicles containing the first two cytokines are polarized at the secretory synapse, while vesicles containing the second two cytokines are scattered throughout the cell (Huse et al., 2006). It would be important to investigate whether WASP is involved in one of these two mechanisms of secretion. Indeed, given the role of WASP

in IS formation, we can speculate that WASP could be also involved in polarized secretion of IL-2 and IFN- γ . However, the defect could be more complex, since WASP deficiency also impairs the synthesis production of IL-2 and IFN- γ .

The acquisition of the ability to produce effector cytokines, including IFN- γ and IL-4 is linked to cell division (Bird et al., 1998). Indeed, the capacity to produce IFN- γ or IL-4 is positively regulated by demethylation of cytokine genes, which increase the accessibility of chromatin to available transcription factors (Agarwal and Rao, 1998). These chromatin modifications are likely to be acquired during cell cycle progression (Richter et al., 1999). We reasoned that our culture conditions, which provide signals through TCR, CD28 and IL-2, should favour cell divisions in WAS and control T cells to a similar extent. Indeed, in our culture system WAS T cells proliferated normally in response to TCR/IL-2. Therefore, impaired production of Th1 cytokines by WAS T cells is likely not to be a consequence of impaired *in vitro* proliferation.

Our finding that in WAS CD4⁺ T cells the production of Th2 cytokines was normal to minimally impaired, while production of Th1 cytokines was strongly defective, could result from an *in vitro* selection of Th2 over Th1-like cells in WAS CD4⁺ T cell cultures. However, WAS CD4⁺ T cells were not intrinsically unable to produce Th1 cytokines, as shown by high levels of IL-2 and IFN- γ produced upon stimulation with TPA plus ionomycin (Figure 9). Moreover, analysis of T-bet and GATA-3 mRNA levels in resting WAS and control CD4⁺ T cell lines argues against a Th2 skewing in WAS cell cultures (Figure 32). Therefore, our results indicate that defective Th1 cytokine production in WAS CD4⁺ T cells results from altered TCR/CD28 signalling. These results were confirmed by the reduced frequency of cells producing IL-2 and IFN- γ , upon TCR/CD28 triggering, in naïve WAS CD4⁺ T cells cultured in Th1-polarizing conditions (Figure 10).

The unbalanced Th2 versus Th1 cytokine production could have at least two possible explanations: the first is that WASP is specifically involved in transmitting the signals leading to the synthesis of Th1 but not Th2 cytokines, the second is that induction of Th1 cytokine gene transcription has a higher threshold than the activation of Th2 cytokine gene transcription. This latest hypothesis has been supported by studies showing that Th1 differentiation and IFN- γ production requires “stronger” or more sustained signalling than the ones required for Th2 and IL-4 differentiation (Constant et al., 1995). This hypothesis is also favoured by the observation that Th2 cytokine secretion was reduced in WAS CD4⁺ T cells with respect to control cells, in case of stimulation with suboptimal anti-CD3 mAb dose (not shown).

Our *in vitro* results are relevant for the understanding of the cellular mechanisms associated WAS immunodeficiency. Reduced IL-2 and IFN- γ production by activated CD4⁺ T cells, and overall impairment in Th1 differentiation is likely to result, *in vivo*, into poor help to CD8⁺ T cells during the establishment of immune response against viral pathogens. The precise requirements of CD4⁺ T cells for the generation and survival of memory CD8⁺ T cells has been deeply investigated (Rocha and Tanchot, 2004). While the activation and acquisition of effector functions by CD8⁺ T cells during primary immune response do not require CD4⁺ help at the moment of priming, the capacity to undergo clonal expansion upon Ag re-exposure is programmed via interaction with CD4⁺ T cells and APC during primary stimulation (Janssen et al., 2003). It has been proposed that help by CD4⁺ cells confers competitive “fitness” to memory CD8⁺ T cells, thus contributing to the generation of long-term surviving memory CD8⁺ T cells (Johansen et al., 2004). We can speculate that, in WAS patients, the inability to efficiently clear viral infections results from both an intrinsic CD8⁺ T cell defect and from inadequate help by Th1 cells. WAS patients often display HSV infections. In healthy individuals, innate and adaptive immunity efficiently control

primary HSV infection. The virus has the ability to persist in the host in a latent state and its reactivation is usually controlled by memory cellular response. Indeed, killing activity and IFN- γ production by virus-specific CD4⁺ and CD8⁺ CTLs were shown to be crucial to control the reactivation phase (Bouley et al., 1995; Rouse and Gierynska, 2001). Therefore, impairment of the Th1-CD8⁺ T cells axis could explain in part the susceptibility to HSV infections in WAS patients, in addition to a defect in NK activity.

Interestingly, we found that CD8⁺ T cell lines established from patient W1 were able to lyse both allogenic EBV-transformed B cells and allogenic PHA-blasts at levels comparable to those of normal donors (Figure 14). Although we cannot exclude an impairment of lytic activity triggered by Ag presented by self MHC, these results suggest that in WAS patients the inability to clear viral infections could be due to poor expansion of Ag-specific CD8⁺ T cells, rather than to defective lytic activity *per se*.

Finally, our data imply a severe impairment in cellular-mediated immunity in WAS patients, which, in addition to a reported defect in NK activity (Gismondi et al., 2004) might be responsible for the high susceptibility not only to viral infections but also to haematological malignancies. The reason for the increased susceptibility of WAS patients to haematological tumours has not been clarified. Immunodeficiency, with lack of immunological surveillance by T cells, plays an important role, as demonstrated by the high percentages of EBV⁺ lymphomas (Imai et al., 2004; Sullivan et al., 1994), however, there could be other causes. Indeed, high incidence of lymphomas in a group of WAS patients carrying a mutation, which is associated with a mild clinical phenotype was reported (Shcherbina et al., 2003). Considering the relatively high frequency of myelodysplasia in WAS patients, it has been proposed that genomic instability contribute to the development of tumours (Burns et al., 2004a). This possibility would be supported by the finding that the yeast-homologue of WASP is involved in the mechanism of cytokinesis (Pelham and Chang, 2002).

5.3 Recruitment of WASP into detergent resistant membranes (DRM) upon T cell activation: a possible association with lipid rafts

The importance of WASP in T cell activation is well established based on the functional defects of WASP deficient human and murine T cells. We and others showed that WASP deficiency impairs the polarization of T cells toward beads coated with anti-CD3/CD28 mAb and the organization of the IS, particularly in conditions of sub-optimal stimulation (Badour et al., 2003; Cannon and Burkhardt, 2004; Dupre et al., 2002). Following studies showed that WASP is recruited to the site of TCR engagement where it is likely to induce localized actin polymerisation (Barda-Saad et al., 2005). We demonstrated that, in normal T cells, early upon TCR/CD28-mediated stimulation, a fraction of WASP re-localizes from soluble fraction to DRM fractions (Figure 15), which have been proposed to correspond to lipid rafts in intact cells (London and Brown, 2000). In accordance with this model, we recovered LAT, a protein which is constitutively associated to rafts, in DRM fractions, independently on T cell activation. These results were subsequently confirmed in an independent study, showing WASP recruitment to DRM (defined as lipid rafts) upon TCR triggering (Sasahara et al., 2002). Partition of WASP to DRM is probably not mediated by Cdc42, as we could never detect Cdc42 in DRM fractions in the same stimulation conditions (not shown). In accordance, binding of Cdc42 was shown to be required for WASP activation at the IS, but not for its localization (Cannon et al., 2001; Zeng et al., 2003).

Following our initial work, the mechanisms regulating WASP association to lipid rafts were further investigated. WASP was found in a complex containing ZAP-70, Lck and Dlg-1, a member of the MAGUK family of proteins (Round et al., 2005). In the same study, Dlg-1 was shown to be recruited to GM1-containing lipid rafts upon T cell activation, likely via interaction with Lck. Interestingly, downregulation of Dlg-1

causes a reduction in the secretion of IL-2 and IFN- γ by T cells, while its overexpression induces an increase of NFAT activity. The accordance between these findings and our results in WASP-deficient T cells suggest that Dlg-1 could influence either WASP localization and/or activation.

An additional study showed that T cell stimulation via TCR and CD2 induced the formation of a complex containing WASP, PSTPIP1, the adaptor protein CD2AP and the costimulatory molecule CD2. In the stimulation conditions used by the authors, WASP was recovered only in non-DRM fractions, suggesting that the mechanisms of WASP partition to DRM may depend on CD28-mediated co-stimulation.

It should be noted that although the inducible partition of WASP to DRM constitutes an indication of the increase of the affinity of the protein for lipid raft microdomains, whether DRM can be really considered the counterpart of rafts in living cells is still a matter of debate. In addition, the extent of partition of a given protein to DRM is strongly influenced by the extraction conditions (Munro, 2003). A formal proof of the association of WASP with specific membrane microdomains (possibly rafts) would ideally come from high-resolution electron microscopy, a system which has been used to investigate dynamic association of signalling molecules with membrane microdomains (Wilson et al., 2004)

5.4 Role of WASP in regulating glycosphingolipids levels: implications for T cell activation

Our data indicate that WASP modulates the levels of GM1 on the surface of T cells (Figure 16 and 17). GM1 up-regulation was functionally linked to T cell activation and to maturation from a naïve to a memory phenotype (Tuosto et al., 2001; Viola et al., 1999). After stimulation through TCR/CD28, reduced levels of GM1 were found on the plasma membrane of WAS T cells, compared to control cells. This reduction was

present both in CD4⁺ and CD8⁺ T cells from patients W1 and W2, although in these patients the defect was more pronounced in CD8⁺ than in CD4⁺ T cells (similarly to what observed in proliferation: Figure 17). Analysis of the total cellular GM1 *versus* the surface one, after TCR/CD28-mediated stimulation, suggested that defective GM1 upregulation in WAS T cells is due both to reduced synthesis (or increased degradation) and reduced translocation of GM1 from intracellular compartments to the plasma membrane. The level of GM1 on plasma membrane of T cells has been correlated with the activation and differentiation status (Tuosto et al., 2001; Viola et al., 1999). Therefore, it was proposed that higher levels of surface GM1 implies higher levels of rafts-associated proteins and a more ready-to-go signalling machinery, which would assure more responsiveness to stimulation. In accordance with this possibility, GM1 was shown to act as a functional co-receptor for the fibroblast growth receptor 2 (FGF2), enhancing the proliferation of CHO cells in response to FGF (Rusnati et al., 2002). Moreover, gangliosides (glycosphingolipids containing sialic acids, as GM1) were found to influence cytokine secretion by T cells (Kanda and Watanabe, 2001). It should be noted, however, that recent studies challenged the importance of the association of signalling molecules to GM1-enriched domains or, more in general to lipid rafts, during T cell activation. One of these study, performed with high-resolution imaging techniques, showed that, in activated T cells, clustered signalling molecules do not necessarily co-localize with clustered lipid rafts, simply because stimulation with anti-CD3/CD28 mAbs does not induce a significant cluster of rafts components (Douglass and Vale, 2005; Glebov and Nichols, 2004). It is then possible that different mechanisms account for molecules segregation during IS formation, including both protein-lipid and protein-protein interactions. In addition, it is possible that different kind of rafts microdomains exist in native membranes, which could have different lipid composition. For this reason, and also to extend the study on GM1 expression, we are

currently investigating the lipid composition of WAS *versus* control T cells by thin layer chromatography (TLC). In the future we would like to extend this kind of analysis to DRM isolated to WAS and control T cells, either in resting or activated state.

In normal T cell lines, inhibition of glycosphingolipids synthesis by the ceramide analogue PDMP (threo-1-phenyl-2-decanoylamino-3-morpholino-1 propanol) did not affect either proliferation or IL-2 secretion in response to TCR/CD28 triggering (Figure 19 and 20). However, a reduction in IL-2 secretion was observed in T cell lines stimulated with immobilized anti-CD3 mAb and treated with PDMP (Figure 19). However, levels of IL-2 elicited by stimulation with anti-CD3 mAb only were in any case very low (<150 pg/ml). Another group obtained similar results in Jurkat T cells, where PDMP treatment was shown to impair signalling mediated by GPI-anchored proteins, but not by the TCR (Nagafuku et al., 2003).

As an overall conclusion, our data imply that reduced GM1 upregulation in WAS T cells is probably the consequence, and not the cause, of their unresponsiveness. However, it is possible that the basal levels of glycosphingolipids influence the responsiveness of T cells to Ag stimulation. In this view, it would be of interest to measure the levels of GM1 and GM3 levels at the plasma membrane of anergic T cells, such as *in vitro* anergized T cells (Schwartz, 2003) or naturally anergic T regulatory cells (Roncarolo et al., 2001a) compared to responsive effector T cells.

5.5 Role of WASP in TCR/CD28-mediated signalling

5.5.1 Defective Th1 cytokine gene transcription in WAS CD4⁺ and CD8⁺ T cells

In this study, we showed that the defects in T cell activation by human WAS CD4⁺ and CD8⁺ T cells resulted in impaired proliferation in response to anti-CD3/CD28 mAb, associated to defective Th1 cytokine production. Analysis of cytokine gene

transcription, after TCR/CD28 triggering, demonstrated that the defective IL-2 and IFN- γ production is due to reduced expression of the corresponding mRNA species (Figure 22 and 23). However, small amounts of IL-2 and IFN- γ mRNA could be produced by WAS CD4⁺ and CD8⁺ T cells, showing that WASP deficiency did not completely block the signalling pathways leading to the transcriptional activation of these cytokine genes. Whether a partial block of transcription is the mechanism responsible for defective Th1 cytokine production in WAS T cells, upon TCR/CD28-mediated stimulation, remains to be determined. It is possible that other functions, such as cell survival or proliferation, influence the secretion of these cytokines. The observation that stimulation with TPA plus ionomycin induced comparable expression of IL-2 and IFN- γ mRNA in WAS and control T cells (Figure 22 and 23) indicates that WAS T cells are not intrinsically unable to activate the signalling pathways, but that they have a specific defect in TCR/CD28-mediated signalling. Interestingly, in WAS CD4⁺ T cells, the levels of IL-4 mRNA were even higher than in control CD4⁺ T cells (Figure 22). Since this finding was not paralleled by an increase in IL-4 secretion by WAS CD4⁺ T cells, we cannot exclude that other post-transcriptional events related to IL-4 synthesis or secretion are affected by WASP deficiency. In different experiments, the relative amount of IL-4 versus IFN- γ mRNA was very different in WAS versus control CD4⁺ T cells. Indeed, in the former the levels of IL-4 and IFN- γ mRNA species were comparable, while in the latter higher IFN- γ than IL-4 mRNA was expressed (Figure 22). These results indicate that the balance between these two cytokines is altered by the absence of WASP.

5.5.2 Defective T-bet induction in WAS CD4⁺ T cells: implications for IFN- γ production and Th1 commitment

Regulation of transcription of IFN- γ or IL-4 in committed Th cells is the result of both epigenetic modifications, which include histone acetylation and DNA methylation at the cytokine gene loci and of acute transcription (Agarwal and Rao,

1998; Avni et al., 2002; Murphy and Reiner, 2002). GATA-3 and T-bet are two crucial factors involved in IL-4 and IFN- γ gene transcription through regulation of the accessibility of cytokine genes, which accompany Th2 or Th1 cell differentiation (Ansel et al., 2003). Our analysis of T-bet and GATA-3 mRNA levels revealed that TCR/CD28-mediated stimulation induced T-bet at lower levels in WAS than in control CD4⁺ T cells, this reduction being more significant at low anti-CD3 mAb dose (Figure 31). Therefore, it is likely that the reduced T-bet expression contributes to the defective IFN- γ gene transcription in WAS CD4⁺ T cells, as T-bet is one of the key factors involved in IFN- γ production by CD4⁺ T cells (Szabo et al., 2000; Szabo et al., 2002) and in Th1 differentiation. T-bet was shown to positively regulate its own expression by a mechanism involving IFN- γ -induced STAT1 activity (Afkarian et al., 2002). Therefore, defective production of T-bet and IFN- γ in WAS CD4⁺ T cells could also result from the lack of a positive feedback. Interestingly, the T-bet defect is less pronounced upon stimulation with higher doses of anti-CD3 mAb (especially in W1 CD4⁺ T cells) and is not present upon TPA plus ionomycin stimulation (Figure 31 and not shown) suggesting that the higher threshold required for activation of WASP-deficient CD4⁺ T cells (Cannon and Burkhardt, 2004; Dupre et al., 2002) could contribute to the reduced ability to express T-bet mRNA. On the other hand, WAS CD4⁺ T cells were able to express normal levels of the transcription factor GATA-3 mRNA (Figure 31), which positively regulate IL-4 gene transcription and Th2 commitment (Ouyang et al., 2000). Although after TCR/CD28 stimulation, we found higher levels of IL-4 mRNA in WAS CD4⁺ T cells, than in control cells (Figure 22), GATA-3 mRNA expression was not enhanced in WAS T cells. These results can be explained by the fact that GATA-3 is not the only factor involved in IL-4 gene transcription (Murphy and Reiner, 2002). In addition, relative levels of T-bet versus GATA-3 are likely to influence the commitment and cytokine production by a T cell, as

T-bet was shown to reduce GATA-3 activity by direct binding (Hwang et al., 2005). Although Th1 cells from WASP knock-out mice displayed reduced IFN- γ secretion (Morales-Tirado et al., 2004), no data were available until present regarding a possible involvement of WASP in the regulation of T-bet and GATA-3 expression. Commitment to Th1 or Th2 phenotype requires signalling through TCR and, in a second moment, through the receptor of polarizing cytokines (Ansel et al., 2003). Human committed Th1 or Th2 cells were shown to re-acquire the ability to produce IL-4 or IFN- γ , respectively, upon culturing in the opposite polarizing conditions (Messi et al., 2003), a property defined as flexibility. It was proposed that irreversible Th1 or Th2 commitment could require the down-regulation of GATA-3 or T-bet, respectively. Based on our data, the balance in IL4 versus IFN- γ levels, secreted by WAS CD4⁺ T cells, upon TCR/CD28 triggering, was strongly in favour of the first cytokine. Therefore, although culture of naïve WAS CD4⁺ T cells in Th1 polarizing conditions, in the presence of anti-IL-4 neutralizing Ab, could confer to WAS cells the acquisition of a Th1-like phenotype (Figure 10), *in vivo* this could not happen due to unbalanced IL-4 versus IFN- γ production and a consequent inability to properly down-regulate GATA-3 expression.

In future studies it will be crucial to measure T-bet and GATA-3 expression during *in vitro* Th1 and Th2 polarization of naïve WAS CD4⁺ T cells. In addition, the possible involvement of defective Th1 responses should be completed by studies *in vivo*, in WASP knock-out mice. These mice could be challenged with pathogens known to induce protective Th1 immune response, such as *Mycobacterium bovis* or *Leishmania major* in the C57BL/6 strain (Murray, 1999; Reiner and Locksley, 1995) and the function of Th1 cells could be tested by their ability to clear infections.

5.5.3 Influence of WASP deficiency on T cell signalling

The above results on cytokine genes transcription strongly suggest that WASP is required to transduce signals from the membrane TCR/CD28 to the cell nucleus. How WASP plays this role has been a matter of debate in the last years. Indeed, although it is now generally accepted that WASP plays an important role in driving actin polymerization and sustaining the formation of IS, it is not clear how the IS modulates downstream events of TCR signalling, such as the activation of transcription factors. We analyzed some of the main signalling events triggered by TCR/CD28 engagements in CD4⁺ and CD8⁺ T cells from WAS patients, using the same stimulation method which previously allowed us to highlight the defects in T cell polarization (Dupre et al., 2002). Our observation that phosphorylation of the MAPK ERK1/2 is generally not affected by WASP deficiency in either CD4⁺ or in CD8⁺ T cells (Figure 24 and 25) is in accordance with previous data obtained in freshly isolated T cells from WASP knock-out mice (Zhang et al., 1999). In addition, we observed normally sustained ERK1/2 phosphorylation. This event was linked to IL-2 and IFN- γ production (Badou et al., 2001; Koike et al., 2003). ERK1/2 MAPK also regulates cell proliferation through direct stimulation of DNA synthesis (Graves et al., 2000). Moreover, in T cells, ERK1/2 is involved in the induction of AP-1 activity by promoting Fos expression (Hunter and Karin, 1992). These activities require translocation of phosphorylated ERK1/2 to the nucleus. Recently, in T cells from WASP knock-out mice, reduced nuclear levels of ERK1/2 have been reported, despite normal phosphorylation, and it has been proposed to be the cause of defective induction of Fos mRNA (Cianferoni et al., 2005). Whether in our system ERK1/2 translocation to the nucleus is affected by WASP deficiency remains to be determined. However, we found no evidence of abnormal levels of nuclear Fos family members in both WAS CD4⁺ and CD8⁺ T cells, stimulated through TCR/CD28 (Figure 28 and 29), which argues against defective ERK1/2 function. This

discrepancy could be due to the different systems considered: T cell lines *versus* freshly isolated T cells. Regarding this possibility, the consistency of defective IL-2 gene transcription in the two systems suggests that T cell lines established from WAS patients retain the main molecular features of freshly isolated T cells. It is however possible that the signalling machinery of our T cells, which have been primed *in vitro*, is different, to some extent, to the one of freshly isolated murine T cells, which are likely to comprise just a small proportion of memory cells. The relevance of our data is supported by the current proposal that WASP deficiency could impair mainly the generation and function of memory T cells (Andreansky et al., 2005).

Similarly to ERK1/2, we found no evidence of reduced phosphorylation of the JNK1/2 MAPKs in WAS CD4⁺ T cells, after TCR/CD28 triggering (Figure 26). JNK activation in T cells requires TCR/CD28 triggering (Su et al., 1994) and it is downstream of the small GTPases Rac1 and Cdc42 (Coso et al., 1995; Minden et al., 1995). Although we cannot exclude that other features of JNK activation (including nuclear translocation) are altered in WAS CD4⁺ T cells, our data do not support a main role of WASP in the regulation of this pathway. In accordance, JNK1/2 are normally phosphorylated in T cells from WASP knock-out mice and the downstream induction of c-Jun mRNA is normal (Cianferoni et al., 2005; Zhang et al., 1999). These results are in accordance with normal TCR/CD28-mediated activation of SAPK/JNK and ERKs in mature T cells from Vav-1 knock-out mice (Fischer et al., 1998). We initially found a modest impairment in Ca²⁺ flux in bulk WAS T cells, upon TCR triggering (Dupre et al., 2002). However, later experiments performed in purified WAS CD4⁺ and CD8⁺ T cells failed to show significant alteration in Ca²⁺ flux (Figure 28). It is possible that these differences are due to the different experimental approaches. Therefore, we provided evidences that in the absence of WASP TCR/CD28 proximal signalling is generally conserved. This finding could challenge the functional relevance of the

reported defects of T cell polarization and IS formation in WASP-deficient T cells (Badour et al., 2003; Cannon and Burkhardt, 2004; Dupre et al., 2002), or even, the role of the IS as the main structure where T cell signalling actually occurs.

Despite the fact that proximal signalling is basically conserved, in CD4⁺ T cells from WAS patients, a reduction in the nuclear levels of NFAT-1, but not NFAT-2, was observed early after TCR/CD28 triggering (Figure 28). Delayed NFAT-1 activation could partially contribute to the defects in IL-2 gene transcription. Similar data have been recently obtained in T cells from WASP knock-out mice, which display defective dephosphorylation and nuclear translocation of NFAT-1, associated to a block in IL-2 gene transcription (Cianferoni et al., 2005). The activation of NFAT by WASP could rely on its binding to WIP. Indeed, WIP was shown to strongly enhance NFAT/AP-1-driven transcription when overexpressed in T cells, together with Vav-1 (Savoy et al., 2000) and moreover, the ability of WASP to potentiate NFAT activity was mapped to its WH1 domain, which comprises the WIP binding site (Silvin et al., 2001). Since it has recently been demonstrated that T-bet positively regulates the binding of NFAT-1 to the IFN- γ promoter by regulating the promoter accessibility (Avni et al., 2002), it is possible that the selective defect in Th1 cytokine production by WAS CD4⁺ T cells results, at least partially, from the combination of NFAT-1 and T-bet defects. It would be important to study the accessibility of IL-2 and IFN- γ promoters in WAS T cells or the *in vivo* binding of transcription factors, such as NFAT by CHIP (chromatin immuno-precipitation).

In WAS CD8⁺ T cells, reduced nuclear levels of both NFAT-1 and NFAT-2 were observed early after TCR/CD28 triggering and could partially account for the defects in cytokine gene transcription. In agreement with our findings, a defect in early NFAT-2 nuclear translocation has been recently reported in NK cells from WAS patients (Huang et al., 2005). Differences observed in CD4⁺ versus CD8⁺ T cells from

WAS patients, in terms of NFATs activation, could be the consequence of intrinsic differences in the threshold required to activate NFAT proteins in these two cell subsets. We have not investigated the expression of T-bet in WAS CD8⁺ T cells. T-bet plays a different role in CD4⁺ T cells, where it is required for IFN- γ production, and in CD8⁺ T cell, where it is dispensable for IFN- γ production (Szabo et al., 2002), but required for perforin-mediated killing. Indeed, in CD8⁺ T cells, T-bet seems to cooperate or act in parallel with eomesodermin, a T-bet homologue, expressed in activated CD8⁺ T cells (Intlekofer et al., 2005). It would be interesting to investigate the expression of these two transcription factors in resting versus activated CD8⁺ T cells from WAS patients.

How WASP deficiency could affect NFAT activation remains to be determined. NFAT nuclear translocation requires dephosphorylation of several serine residues by calcineurin, which is activated by Ca²⁺/calmodulin (Hogan et al., 2003), however we found no differences in the Ca²⁺ flux in WAS *versus* control T cells. It is possible that an inability to sustain Ca²⁺ flux for longer times may be responsible for defective NFAT activation in WAS T cells. The analysis of TCR-mediated Ca²⁺ flux in human and murine WASP-deficient T cells gave contrasting results (Henriquez et al., 1994; Molina et al., 1993; Zhang et al., 1999). One possible explanation for these discrepancies is that WASP deficiency causes only very mild alteration in TCR-mediated Ca²⁺ flux, which can be identified or not, with a strong dependency on the experimental conditions. However, NFAT activation was shown to be regulated also by post-translational modification, including phosphorylation and sumoylation (Chow et al., 2000; Terui et al., 2004). Therefore, defective NFAT1 and NFAT-2 activation in WAS T cells could be due either to reduced, or less sustained, Ca²⁺ flux or to alteration of other signalling pathways.

Analysis of I κ B α phosphorylation in WAS CD4⁺ T cells, after CD3/CD28 triggering, showed that at late time points the levels of phosphorylation were higher

than in control cells (Figure 30). I κ B α is normally degraded after phosphorylation by the proteasome pathway (Karin and Ben-Neriah, 2000). At the later time point evaluated (2 h) higher levels of I κ B α phosphorylation could reflect either reduced degradation or increased re-synthesis of I κ B α . In T cells from WASP knock-out mice, activity of NF κ B was found to be normal (Cianferoni et al., 2005). Costimulation of T cells through CD28 is necessary to induce NF κ B activation, which is involved in the transcription of IL-2 (Ghosh et al., 1993). NF κ B activity was shown to be regulated by Vav-1, possibly by direct interaction with members of the NF κ B complex (Piccolella et al., 2003). In addition, the activation of NF κ B has been shown to occur upon recruitment to the IS, at the level of rafts (Gaide et al., 2002; Wang et al., 2004). Therefore it would be interesting to further evaluate NF κ B activity and localization of the various NF κ B subunit (including p65 and c-Rel) upon stimulation with anti-CD3/CD28 mAb-coated beads.

This study demonstrates that T cells from WAS patients displayed alteration in the activation of various transcription factors, as it was shown in previous studies in different cellular models (Badour et al., 2004a; Cannon and Burkhardt, 2004; Cianferoni et al., 2005; Huang et al., 2005). These results suggest that rather than controlling a single signalling pathway downstream the TCR/CD28, WASP may regulate the threshold for activation of different pathways converging towards the transcriptional induction of cytokine genes. In addition, we can speculate that the influence of WASP on the transcriptional regulation of T cells could be partially due to a possible translocation of WASP itself from the cytosol to the nucleus. Such a mechanism, indeed, has been demonstrated for the WASP homologue N-WASP, whose nuclear versus cytosolic localization has been shown to influence the expression of the heat shock protein HSP90 and, by a feedback mechanisms, the activity of Src kinase

(Suetsugu and Takenawa, 2003). This possibility could be tested by analyzing the sub-cellular localization of WASP in resting *versus* activated T cells.

In addition, WASP-deficient cells represent a useful model to study the connection of T cell polarization and IS formation to downstream TCR-related events, such as the activation of transcription factors. We showed that WASP deficiency impairs both lipid rafts dynamics and activation/expression of transcription factors, upon TCR/CD28 triggering. In the future, a single-cell analysis, performed by living microscopy, could be envisaged in the WAS model, or in other models of defective IS formation, in order to define the precise functional and temporal links between IS organization, and activation of transcription factors.

5.6 The role of WASP in nTreg function

Results obtained from the study of nTreg cells from WAS patients (Figure 33 and 34), as well as of nTreg cells from WASP knock-out mice (Marangoni F. *Personal communication*), suggest that defective nTreg function could contribute to the high susceptibility to autoimmune manifestations reported in WAS patients (Dupuis-Girod et al., 2003). The observation that the suppressive function of human WAS nTreg was partially abrogated, suggests that either the frequency of “bona fide” nTreg cells among the CD4⁺/CD25^{bright} population is lower in WAS patients than in healthy donors, or that nTreg cells are not properly activated. Regarding the first hypothesis, the frequency of FoxP3⁺ cells among CD4⁺ cells in the peripheral blood of WAS patients was found to be elevated (Figure 32B). FoxP3 was shown to be expressed in human CD4⁺/CD25⁺ nTreg cells and also in recently activated CD4⁺/CD25⁻ effector T cells (Allan et al., 2005; Walker et al., 2003). We have no information on the proportion of nTreg cells in secondary lymphoid organs of WAS patients, but a parallel study performed in our laboratory showed normal percentages of nTreg cells in the spleen of WASP knock-out

mice (Marangoni F. et al, *Personal communication*). Our data suggest that the defective suppressive ability of WAS nTreg cells is due to impaired function. However, a more wide analysis of CD25 versus FoxP3 expression levels on CD4⁺ T cells will clarify whether a difference could also exist in the percentages of cells expressing CD25 only, FoxP3 only, or both CD25 and FoxP3, in WAS patients versus age-matched healthy donors.

Several reports indicated IL-2 as a crucial, non-redundant factor, able to drive maturation and function of nTreg cells, both *in vitro* and *in vivo* (Malek and Bayer, 2004). Indeed, an intact IL-2/IL2R signalling was shown to be required for nTreg development (Bayer et al., 2005; Malek et al., 2000). However, recent findings indicate that IL-2-mediated signals are not strictly required for the maturation of murine nTreg cells, identified by the expression of FoxP3. IL-2 has been rather proposed to be required for the survival of nTreg cells in the periphery and for the maintenance of FoxP3 expression (Fontenot et al., 2005b). In WAS patients, IL-2 defects seems not to affect the generation of nTreg cells. Therefore, either IL-2 is dispensable for nTreg generation in humans, or, more likely, IL-2 could be produced in sufficient amounts in WAS patients to drive nTreg development. In addition, it is conceivable that, in addition to T cells, other cells in the thymus could provide IL-2 to the developing nTreg. For example, DC have been shown to drive positive selection of nTreg in thymic Hassall's corpuscles (Watanabe et al., 2005).

Although nTreg cells are anergic *in vitro* (but not *in vivo*) (von Boehmer, 2003) their ability to suppress requires signalling through the TCR and the presence of IL-2 (Thornton et al., 2004a). Although nTreg actively suppress IL-2 mRNA transcription by effector T cells, it was proposed that low levels of IL-2, produced in a first phase by effector T cells, are sufficient to sustain nTreg suppressive activity, without reversing their anergy (Thornton et al., 2004b). Since WASP lowers the threshold for TCR-

mediated T cell activation, it is possible that WAS nTreg cells require stronger stimulation than normal nTreg cells to suppress at comparable levels. This possibility is supported by the observation that the defective suppressive activity of nTreg cells from WASP knock-out mice was more pronounced when suppression was carried out with low dose of anti-CD3 mAb (Marangoni F., *Personal communication*). In addition, proliferative response of murine WAS nTreg cells to anti-CD3/CD28 mAb was strongly defective, supporting the hypothesis of impaired activation. We have preliminary data suggesting that nTreg cells from WAS patients respond less efficiently, in terms of proliferation, than control nTreg to TCR/CD28/IL-2-mediated signals (not shown). This finding differs from the results obtained in WAS effector T cells, which proliferate normally in response to high dose of IL-2 plus anti-CD3/CD28 stimulation. This difference could be explained by the observation that murine nTreg cells seem to have a higher threshold for IL-2-driven proliferation than effector T cells (Thornton et al., 2004b). We can speculate that in a context of high threshold for activation, IL-2-induced signalling and proliferation in WAS nTreg could be affected in a way similar to TCR-induced signalling and proliferation in effector T cells. This hypothesis could be investigated by evaluating IL-2 dose-response, with concomitant TCR triggering, of murine WASP knock-out nTreg cells.

Finally, another relevant issue in the context of WAS could be the repertoire of nTreg cells. A recent study proposed that in wild type mice a subset of non regulatory T cells express self reactive TCRs which are shared also by a fraction of nTreg cells (Hsieh et al., 2006). Therefore, the shaping of the TCR repertoire seems to be crucial in the homeostasis of nTreg versus auto-reactive T cells. In WAS patients, skewing of TCR repertoire was shown starting from the 15 years of age (Wada et al., 2005). This could have important implications in the development of autoimmunity on one side, and of cancer, on the other. Analysis of the TCR repertoire of nTreg and non regulatory T

cells in WASP knock-out mice could provide useful additional information on the role played by nTreg cells in WAS-associated autoimmunity. However, the most relevant findings would arise only from the study of nTreg cells repertoire in WAS patients, as this repertoire would be shaped by the natural history of infections and eventually autoimmunity experienced by the patient.

6 CONCLUDING REMARKS

The results presented in this thesis, show that WASP regulates the threshold for T cell activation. We demonstrated that WASP is recruited to DRM upon T cell activation and that its deficiency impairs GM1 upregulation upon TCR/CD28 triggering. Although WASP deficiency does not significantly affect proximal TCR/CD28 signalling, it alters the transcriptional activation of Th1 cytokine genes in CD4⁺ and CD8⁺ T cells. The reduced activation of NFAT, especially in CD8⁺ T cells, and the defective induction of T-bet transcription factors, in CD4⁺ T cells, could contribute to the defects of cytokine gene transcription observed in T cells from WAS patients. The defects in cellular immunity here identified help to clarify the mechanisms underlying WAS-associated immunodeficiency, characterized by high susceptibility towards infections and tumours. Finally, we propose that a defect in the suppressive function of nTreg cells contributes to the development of autoimmune manifestations in WAS patients.

Altogether, these data help to clarify the role of WASP in T cell activation and regulation and in the mechanisms implicated in WAS pathogenesis. In addition, they provided the basis for a future gene therapy approach in this genetic disease.

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